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Progesterone receptor gene expression is induced by estrogen in MCF-7 human breast cancer cells. Although it is generally thought that estrogen-responsiveness is mediated through estrogen response elements (EREs), the progesterone receptor gene lacks an identifiable ERE. The progesterone receptor A promoter does, however, contain a half ERE/Sp1 binding site comprised of an ERE half site upstream of two Sp1 binding sites. We have used *in vivo* DNase I footprinting to demonstrate that the half ERE/Sp1 binding site is more protected when MCF-7 cells are treated with estrogen than when cells are not exposed to hormone suggesting that the this region is involved in estrogen-regulated gene expression. The ability of the half ERE/Sp1 binding site to confer estrogen responsiveness to a simple heterologous promoter was confirmed in transient cotransfection assays. *In vitro* DNase I footprinting and gel mobility shift assays demonstrated that Sp1 present in MCF-7 nuclear extracts and purified Sp1 protein bound to the two Sp1 sites and that estrogen-occupied estrogen receptor enhanced Sp1 binding. In addition to its effects on Sp1 binding, the estrogen receptor also bound directly to the ERE half site. Taken together, these findings suggest that estrogen-occupied receptor and Sp1 play a role in activation of the human progesterone receptor A promoter

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INTRODUCTION

The progesterone receptor (PR) gene is under estrogen control in normal normal mammary cells and in MCF-7 human breast cancer cells (Nardulli et al. 1988; Read et al. 1988; Wei et al. 1988). MCF-7 PR mRNA and protein increase and reach maximal levels after three days of 17 β-estradiol (E_2) treatment (Nardulli et al. 1988; Read et al. 1988; Wei et al. 1988). Two distinct PR forms are differentially expressed in a tissue-specific manner (Horwitz and Alexander 1983; Mohamed et al. 1994; Schrader and O'Malley 1972; Tung et al. 1993; Vegeto et al. 1993). PR-B is a 120 kD protein containing a 164 amino acid amino-terminal region that is not present in the 94 kD PR-A. Two discrete promoters, A and B, which are responsible for the production of PR-A and PR-B, respectively, have also been defined (Kastner et al. 1990). The activities of these two promoters are increased by estrogen treatment of transiently transfected Hela cells. Interestingly, no consensus estrogen response elements (EREs) have been identified in either Promoter A (+464 to 1105) or Promoter B (-711 to +31). Promoter A does, however, contain an ERE half site located upstream of two Sp1 sites (Kastner et al. 1990). The presence of these adjacent binding sites suggests that the ER might be able to influence PR expression directly by binding to the ERE half site, indirectly by interacting with proteins bound to the putative Sp1 sites, or a combination of these two methods. To determine whether the ERE half site and the two Sp1 sites present in the human PR A promoter might impart estrogen responsiveness to the PR gene, a series of *in vivo* and *in vitro* experiments were carried out.

BODY

In vivo footprinting is an extremely challenging procedure. The design of primers and the isolation of purified DNA samples are critical to the success of the overall procedure. In addition, it is crucial that the sequence adjacent to an area of interest does not contain substantial secondary sequence so that the polymerase can readily move through the intervening nucleotide sequence. Because these combined factors can limit one's ability to successfully carry out *in vivo* footprinting, we completed fewer *in vivo* footprinting studies that originally outlined in the original proposal. Thus, Specific Aim 1, Determine the effects of estrogen treatment on protein-DNA interactions in MCF-7 cells, was examined and the results of these studies follow. However, Specific Aim 2 , Determine the effects of concurrent estrogen and antiestrogen treatment on protein-DNA interactions in MCF-7 cells and Specific Aim 3, Examine the PR gene in the hormone-insensitive MDA-MB-231 cells were not addressed. Rather, we chose to concentrate on an ERE half site adjacent to two Sp1 sites, which was protected in *in vivo* footprints and complement these *in vivo* experiments with *in vitro* assays. In so doing, we were able to not only define a region involved in estrogen-regulated transcription of the PR gene, but also to identify factors involved in this regulation. These complementary studies provide us with a better understanding of how this gene is regulated in human breast cancer cells.

Experimental Procedures

Cell Culture. MCF-7 human breast cancer cells (Soule et al. 1973) were maintained in Eagle's Minimum Essential Medium (MEM) containing 5% heat-inactivated calf serum. Cells were seeded in 10 cm plates and transferred to phenol red free, serum free Improved MEM

(Katzenellenbogen and Norman 1990) five days before the experiments were conducted. Chinese Hamster Ovary (CHO) cells were maintained in DMEM/F12 supplemented with 5% charcoal dextran stripped calf serum (Eckert and Katzenellenbogen 1982).

Oligonucleotides and Plasmid Constructions. The names and sequences of wildtype (wt) or mutant half ERE/Sp1 binding site are listed. Nucleotides that differ from the endogenous, wt half ERE/Sp1 binding site are underlined.

ERE/Sp1 wt: 5'-GATCTAGGAGCTGACCAGCGCCGCCCTCCCCCGCCCCCGACCA-3'

and 5'-GATCTGGTCGGGGGCGGGGGAGGGCGGCGCTGGTCAGCTCCTA-3',

ERE/Sp1 mP/D: 5'-GATCTAGGAGCTGACCAGCGTTGTACTCCCTTGTACCCGACCA-3'

and 5'-GATCTGGTCGGGTACAAGGGAGTACAACGCTGGTCAGCTCCTA-3',

ERE/Sp1 mD: 5'-GATCTAGGAGCTGACCAGCGTTGTACTCCCCCGCCCCCGACCA-3'

and 5'-GATCTGGTCGGGGGCGGGGGAGTACAACGCTGGTCAGCTCCTA-3',

ERE/Sp1 mP: 5'-GATCTAGGAGCTGACCAGCGCCGCCCTCCCTTGTACCCGACCA-3'

and 5'-GATCTGGTCGGGTACAAGGGAGGGCGGCGCTGGTCAGCTCCTA-3',

ERE/Sp1 mE: 5'-GATCTAGGAGCTGATTAGCGCCGCCCTCCCCCGCCCCCGACCA-3'

and 5'-GATCTGGTCGGGGGCGGGGGAGGGCGGCGCTAATCAGCTCCTA-3'.

ERE/Sp1 wt oligos with Bgl II compatible ends were subcloned into the *Bgl* II-cut, dephosphorylated chloramphenicol acetyl transferase (CAT) reporter plasmid, TATA CAT (Chang et al. 1992), to create ERE/Sp1-TATA CAT. The ligated vector was transformed into the DH5α strain of *E. coli*, sequenced, and purified on two cesium chloride gradients.

In vitro and *in vivo* treatment of genomic DNA. MCF-7 cells were exposed to ethanol vehicle or 1 nM E_2 for 0, 2 ,or 72 hours prior to DNase I treatment. Cells were permeabilized with 0.4%

NP-40 and treated with 750 U DNase I / ml (Boehringer Mannheim, Indianapolis, IN) for 3 min at 25°C. Isolation of genomic DNA was carried out as described by Mueller and Wold (Mueller and Wold 1992). The genomic DNA was purified, incubated with RNase A, resuspended in TE (10mM Tris pH 7.5, 1mM EDTA) and stored at -20 °C.

Naked genomic DNA was treated *in vitro* with dimethylsulfate (DMS) as described (Mueller and Wold 1992). *In vitro* DNase I-treated DNA was prepared by adjusting 100 µg of protein-free, RNase A-treated DNA to 175 µl with TE. DNA was incubated with 2.5×10^{-5} U DNase I for 5 min at 37° C. The reaction was stopped by the addition of 10 mM EDTA, and processed as described for *in vivo*-treated genomic DNA.

In vivo footprinting. Ligation mediated PCR (LMPCR) footprinting was carried out essentially as described by Mueller and Wold (Mueller and Wold 1989; Mueller and Wold 1992). 2 µg of genomic DNA was subjected to LMPCR procedures using nested primers, which annealed to sequences upstream of the half ERE/Sp1 binding site (+571 to +595) in the human PR gene. The primer sequences were: Primer 1- 5'TCCCCGAGTTAGGAGACGAGAT3', Primer 2- 5'CGCTCCCCACTTGCCGCTC3', and Primer 3- 5'GCTCCCCACTTGCCGCTCGCTG3'. The annealing temperatures for the primers were 55°, 62°, and 69°, respectively. The linker primers LMPCR 1 and LMPCR 2 described by Mueller and Wold (Mueller and Wold 1989) were also used, except that LMPCR 1 was modified by removing the two 5' nucleotides to eliminate potential secondary structure.

In vitro DNase I footprinting. Primers, which annealed 88 bp upstream (Primer 3) or 79 bp downstream (Primer 4-5'TCGGGAATATAGGGGCAGAGGGAGGAGAA3') of the half ERE/Sp1 binding site, were subjected to 30 rounds of PCR amplification with 30 ng of the PR-

(+464/+1105) CAT (Kastner et al. 1990). Labeling of the coding and noncoding strands was carried out with ^{32}P-labeled Primer 3 or Primer 4, respectively. The 181 bp singly end-labeled amplified fragments were fractionated on an acrylamide gel and isolated. End-labeled DNA fragments (100,000 cpm) containing the half ERE/Sp1 binding site were incubated for 15 min at room temperature in a buffer containing 10% glycerol, 50 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, 1 mM $MgCl_2$, 50ng of poly dIdC and 0.4 mM DTT in a final volume of 50 µl with either 30-60 µg of MCF-7 nuclear extract, 12.5-37.5 ng of purified Sp1 protein (Promega, Madison, WI) or 15 ng of purified Sp1 and 25-100 fmol of purified Flag-tagged ER, which had been expressed and purified as described by Kraus and Kadonaga (Kraus and Kadonaga 1998). 10 nM E_2 was included in binding reactions containing the purified ER. Bovine serum albumin (BSA) was included with the purified Sp1 protein or the purified Sp1 and ER so that the total protein concentration in each reaction was 25 µg. When MCF-7 nuclear extracts were used, ovalbumin and KCl were added as needed to maintain constant protein and salt concentrations and poly dI/dC was increased to 1 µg per reaction. 1 - 2 U of RQ1 ribonuclease-free DNase I (Promega, Madison, WI) was added to each sample and incubated at room temperature for 0.75- 8 min. The DNase I digestion was terminated by addition of stop solution (200mM NaCl, 1% SDS, 30 mM EDTA and 100 ng/µl tRNA) The DNA was phenol/chloroform extracted, precipitated, and resuspended in formamide loading buffer (Chodosh 1989). Samples were fractionated on an 8% denaturing acrylamide gel. Radioactive bands were visualized by autoradiography and quantitated with a Molecular Dynamics phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Gel mobility shift assays. Gel mobility shift assays were carried out essentially as described

(Nardulli et al. 1991; Petz et al. 1997). ^{32}P-labeled (10,000 cpm) half ERE/Sp1-containing wild type or mutant oligos were incubated for 15 min. at room temperature in a buffer containing 10% glycerol, 50 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, 1 mM $MgCl_2$, 50ng of poly dI/dC and 0.4 mM DTT in a final volume of 20 µl with either 20 µg of MCF-7 nuclear extract, 0.25-3 ng of purified Sp1 protein, or 0.25 ng of purified Sp1 and 5-40 fmol of purified ER. 10 nM E_2 was included in all binding reactions containing ER. BSA was included when purified Sp1 or ER were used so that the total protein concentration in each reaction was 20 µg. When MCF-7 nuclear extracts were used, the nonspecific DNA for each reaction included 1 µg of salmon sperm DNA and poly dI/dC was increased to 2 µg. For antibody supershift experiments, the Sp1-specific monoclonal antibody, 1C6 (Santa Cruz Biotech, Santa Cruz, CA) or the ER-specific monoclonal antibody, H222, (Kindly provided by Dr. Geoffrey Greene, University of Chicago, Chicago, IL) was added to the protein-DNA mixture and incubated for 10 min at room temperature. Low ionic strength gels and buffers were prepared as described (Chodosh 1989). Radioactive bands were visualized by autoradiography.

Transient transfection of CHO cells. CHO cell transfections were performed using the calcium phosphate method (Nardulli et al. 1995). Crystals were formed in the presence of 3 µg of the indicated CAT reporter, 200 ng of the β-galactosidase vector pCH110 (Pharmacia, Piscataway, NJ), 5 ng of the human ERα expression vector pCMVhER (Reese and Katzenellenbogen 1991), and 4.8 µg of pTZ18U and incubated with CHO cells for 16 hrs followed by a 2 min 20% glycerol shock. Cells were maintained in media containing ethanol vehicle or 10 nM E_2 for 24 hrs. Protein concentration was determined using Bio-Rad (Hercules, PA) protein assay with BSA as a standard. Mixed-phase CAT assays were performed using 35 µg protein as previously described

(Nielsen et al. 1989). The β-galactosidase activity was determined at room temperature as previously described (Herbomel et al. 1984) and used to normalize the amount of CAT activity in each sample.

Results

In vivo footprinting of the PR gene. A number of studies have suggested that an Sp1 site alone or in combination with an imperfect ERE or ERE half site may be involved in conferring estrogen responsiveness to target genes (Dubik and Shiu 1992; Krishnan et al. 1994; Porter et al. 1997; Porter et al. 1996; Rishi et al. 1995; Scholz et al. 1998; Wu-Peng et al. 1992). To determine whether the ERE half site and two potential Sp1 sites residing in the endogenous human PR gene (+571 to +595, Ref. (Kastner et al. 1990) might be involved in estrogen-regulated transactivation, *in vivo* DNase I footprinting was carried out using MCF-7 cells. The region of the PR A promoter containing the consensus ERE half site and two potential Sp1 sites is shown in Fig. 1 and will be hereafter referred to as the half ERE/Sp1 binding site.

To carry out the *in vivo* footprinting assays, MCF-7 cells were treated with ethanol vehicle or with E_2 for 2 or 72 hours and then exposed to DNase I. The cells were rapidly lysed, DNA was isolated, and LMPCR procedures were carried out (Mueller and Wold 1992). Naked genomic DNA, which had been treated *in vitro* with DNase I served as a reference in identifying sequences that were susceptible to cleavage in the absence of proteins (Fig. 2, V_t). When cells were treated with E_2 for 2 hours, the protection of the proximal Sp1 site ($Sp1_P$), the distal Sp1 binding site ($Sp1_D$), and the ERE half site was greater than seen in cells that had not been exposed to hormone. After 72 hours of E_2 treatment, a time when PR mRNA and protein reach maximal

levels (Eckert and Katzenellenbogen 1982; Graham et al. 1996; Nardulli et al. 1988; Read et al. 1988; Wei et al. 1988), the protection of the half ERE/Sp1 site was sustained. E_2 treatment also elicited protection of regions flanking the half ERE/Sp1 binding site. Thus, we were able to detect distinct differences in protection of the half ERE/Sp1binding site on the coding strand of the endogenous PR gene after E_2 treatment. Despite numerous attempts, we were unable to obtain a footprint of the noncoding PR DNA strand in this region. The failure of the LMPCR reactions may have been due to formation of an extensive stem loop structure (ΔG = - 11.5 Kcal/mol) extending from + 674 to + 733 (Kastner et al. 1990) that limited primer annealing or interfered with the ability of polymerase to proceed through this region. None the less, our *in vivo* footprinting of the coding strand demonstrated that the half ERE/Sp1 binding site residing in the endogenous PR gene was differentially protected in ethanol- and E_2-treated MCF-7 cells and suggested that the ERE half site as well as the proximal and distal Sp1 sites might be involved in regulation of the endogenous PR gene in MCF-7 cells.

Estrogen enhances transcription of a reporter plasmid containing the half ERE/Sp1 binding site.

To determine if the half ERE/Sp1 binding site could confer estrogen-responsiveness to a heterologous promoter, transient cotransfection experiments were carried out with a human ER expression vector and a CAT reporter plasmid containing either a TATA box (TATA CAT) or the half ERE/Sp1 binding site and a TATA box (ERE/Sp-TATA CAT). Exposure of transiently cotransfected CHO cells to E_2 resulted in an increase in CAT activity when the reporter plasmid contained the half ERE/Sp1 binding site (Fig. 3 ERE/Sp1-TATA CAT). In contrast, no change in activity was observed with E_2 treatment when the parental reporter plasmid containing a TATA box was used (TATA CAT). These findings suggest that the half ERE/Sp1 binding site is

involved in estrogen-mediated activation of the PR A promoter.

Proteins present in MCF-7 nuclear extracts bind to the half ERE/Sp1 binding site *in vitro*. Our *in vivo* footprinting and transient transfection experiments provided evidence for the involvement of the half ERE/Sp1 binding site in mediating estrogen's effects on the PR A promoter. However, these studies did not allow us to identify proteins that interact with this DNA sequence. To begin to identify proteins that bind to this site, gel mobility shift assays were carried out with MCF-7 nuclear extracts. When ^{32}P-labeled oligos, each containing the half ERE/Sp1 binding site, were combined with nuclear extracts prepared from E_2-treated MCF-7 cells, one major protein-DNA complex was formed (Fig. 4, Lane 1). Since we anticipated that ER and Sp1 might bind to this region, antibodies to these proteins were included in separate binding reactions. The major protein-DNA complex was supershifted by the Sp1-specific antibody 1C6, which binds only to Sp1 and does not cross react with Sp2-4 (Lane 2). In contrast, the major protein-DNA complex was not affected by the ER-specific antibody H222 (Lane 3). These data indicate that Sp1 was present in substantial amounts in our MCF-7 nuclear extracts and that it bound efficiently to the half ERE/Sp1 binding site. However, these experiments did not provide evidence that the ER was involved in formation of the protein-DNA complex.

Gel mobility shift experiments require the formation of stable protein-DNA complexes, which must be maintained during extended periods of electrophoresis. To determine whether more transient or lower affinity interaction might occur between MCF-7 nuclear proteins and the ERE half site and/or either one or both of the Sp1 binding sites, *in vitro* DNase I footprinting was carried out. 181bp DNA fragments, each containing the half ERE/Sp1 binding site and additional PR flanking sequence, were ^{32}P-labeled on one end, incubated with increasing amounts of MCF-7

nuclear extract, and exposed to limited DNase I cleavage (Fig. 5, Lanes 3-5 and 8-10). When DNA fragments, which had been ^{32}P -labeled on the coding strand were utilized, the proximal and distal Sp1 sites were partially protected by proteins present in the MCF-7 nuclear extracts (Lanes3-5). Quantitative analysis of the coding strand revealed slightly greater protection of the proximal Sp1 site than the distal Sp1 site. Although the ERE half site was not protected, nucleotides within and immediately flanking the ERE half site were hypersensitive to DNase I cleavage upon addition of increasing concentrations of nuclear proteins (Lanes 3-5). When the noncoding DNA strand was labeled and utilized in *in vitro* footprinting experiments with MCF-7 nuclear extracts, the proximal Sp1 site was more extensively protected than the distal Sp1 site (Lane 8-10). As seen with the coding strand, hypersensitive sites were observed within and adjacent to the ERE half site on the noncoding strand. Control lanes containing DNA fragments, which had been exposed to DMS (Lanes 1 and 6) or DNase I (Lanes 2 and 7) in the absence of protein, were included for reference. The enhanced protection of the Sp1 sites observed in our *in vitro* footprints in the presence of MCF-7 nuclear extracts was similar to the increased protection of the Sp1 sites in the endogenous gene upon E_2 treatment of MCF-7 cells. The ERE half site was not protected in our *in vitro* footprints as seen in the *in vivo* footprints, but rather displayed hypersensitivity to DNase I cleavage on both strands. Since DNase I hypersensitivity can result from binding of a protein to the major groove of the DNA helix making the minor groove more accessible to DNase I cleavage (Suck 1994), the hypersensitivity observed within and adjacent to the ERE could result from binding of a protein to the major groove in the region of the ERE.

Purified Sp1 binds to the half ERE/Sp1 binding site. Our antibody supershift experiments indicated that native Sp1 present in MCF-7 nuclear extracts was binding to the half ERE/Sp1

binding site. However, the MCF-7 extracts used in these assays contained a complex combination of nuclear proteins. To determine whether the Sp1 protein alone was capable of binding to the half ERE/Sp1 binding site or whether other proteins present in the MCF-7 nuclear extracts were required for Sp1 binding, gel mobility shift experiments were carried out with purified Sp1 protein. ^{32}P-labeled oligos, each containing the half ERE/Sp1 binding site, were incubated with increasing concentrations of purified Sp1 protein and fractionated on a nondenaturing acrylamide gel (Fig. 6, Lanes 2-5). At the lowest Sp1 concentration utilized (1 ng), a single gel-shifted band was observed (←1, Lane 2). As increasing concentrations of Sp1 were added to the binding reaction, there was a dose-dependent increase in a second, higher molecular weight complex (←2, Lanes 3-5). These experiments demonstrate that purified Sp1 was capable of forming a stable complex with the half ERE/Sp1 binding site. Additional gel shift assays demonstrated that the more rapidly migrating Sp1-DNA complex had the same mobility as the complex formed with MCF-7 nuclear extracts (Data not shown).

It seemed likely that the formation of the higher order complex in our gel shift experiments represented the simultaneous binding of two Sp1 proteins to the two Sp1 sites and the more rapidly migrating complex represented Sp1 binding to one of the two Sp1 sites. To determine if Sp1 was binding to one or both of the Sp1 sites and whether it displayed any preference in binding to the proximal or the distal Sp1 site, *in vitro* footprinting experiments were carried out. 181 bp DNA fragments, each containing the half ERE/Sp1 binding site, were ^{32}P-labeled on the coding strand and incubated with increasing concentrations of purified Sp1 protein. When 12.5 ng of purified Sp1 was included in the binding reaction, the proximal and distal Sp1 sites were protected (Fig. 7, Lanes 3). Addition of 25 and 37.5 ng of purified Sp1 protein further enhanced protection

of the two Sp1 sites (Lanes 4-5). When DNA fragments labeled on the noncoding strand were incubated with increasing amounts of purified Sp1, the proximal Sp1 site was more protected than the distal Sp1 site (Lanes 8-10). This preference for the proximal Sp1 site was also evident in the *in vitro* footprints of the noncoding strand in the presence of MCF-7 nuclear extracts (Fig. 5). Control lanes containing DNA fragments, which had been exposed to DMS (Fig. 7, Lanes 1 and 6) or DNase I (Lanes 2 and 7) in the absence of proteins, were included for reference. These data combined with our gel mobility shift assays supports the idea that Sp1 binds first to the proximal Sp1 site and then to the distal Sp1 site.

ER enhances Sp1 binding to the half ERE/Sp1binding site. Our *in vitro* binding assays suggested that Sp1 was involved in regulating the PR gene, but left some question about the involvement of ER in this process. From previous studies examining ER-mediated transcription activation, it seemed possible that ER could increase transcription either directly by binding to the ERE half site or indirectly by enhancing Sp1 binding (Duan et al. 1998; Dubik and Shiu 1992; Krishnan et al. 1994; Porter et al. 1997; Porter et al. 1996; Rishi et al. 1995; Wang et al. 1998; Wu-Peng et al. 1992). To determine if ER affected protein-DNA complex formation, gel mobility shift assays were carried out. When ^{32}P-labeled oligos, each containing the half ERE/Sp1 binding site, were incubated with 3 ng of purified Sp1 (Fig. 8A, Lane 1), a single gel shifted band was observed. When the amount of purified Sp1 protein was decreased to 0.25 ng, a faint gel shifted band was barely visible (Lane 2). As 5-40 fmol of purified, E_2-occupied ER was added to 0.25 ng purified Sp1 protein, an increase in Sp1 binding was observed (Lanes 3-6). Addition of 40 fmoles of ER increased Sp1 binding 13.1 ($\pm$ 4.2 SE)-fold in three separate experiments. This increased binding was not due to an increase in protein concentration since all reactions contained the same amount

of total protein. Interestingly, ER enhanced Sp1 binding, but did not change the mobility of the protein-DNA complex indicating that the ER was not present in the complex. The ability of ER to enhance Sp1 binding without forming a trimeric ER-Sp1-DNA complex in gel mobility shift assays has been noted by others (Porter et al. 1997; Wang et al. 1998). Addition of increasing amounts of E_2-occupied ER to the binding reaction also produced a dose-dependent increase in a second, more rapidly migrating complex, which we thought most likely resulted from ER binding to the ERE half site. To confirm which protein-DNA complexes contained ER and Sp1, antibody supershift experiments were carried out. Addition of ER and Sp1 to the binding reaction resulted in the formation of two protein-DNA complexes (Fig. 8B, Lane 7). The Sp1-specific antibody 1C6 supershifted the more slowly migrating complex, but did not affect the more rapidly migrating complex (Lane 8). The ER-specific antibody H222 decreased the intensity of the more rapidly migrating complex, but did not affect the Sp1-DNA complex (Lane 9). The abilities of these antibodies to interact specifically with the Sp1-DNA and ER-DNA complexes was demonstrated using either purified Sp1 in the absence of ER (Lanes 1-3) or purified ER in the absence of Sp1 (Lanes 4-6). These antibody supershift experiments confirmed that the more slowly migrating complex contained Sp1 and the more rapidly migrating complex contained ER. In contrast to these findings with the E_2-occupied ER, addition of unoccupied ER to levels as high as 100 fmoles, failed to enhance Sp1 binding (Data not shown). Thus, the addition of purified E_2-occupied ER to the binding reaction not only enhanced Sp1 binding, but also resulted in ER binding, presumably, to the ERE half site.

To determine how ER affected Sp1 protection of the half ERE/Sp1 binding site, *in vitro* DNase I footprinting experiments were carried out with purified ER and Sp1 proteins. When 15

ng of purified Sp1 was incubated with the ^{32}P-labeled coding strand, the proximal and distal Sp1 sites were protected (Fig. 9, Lanes 3). Addition of 15 ng Sp1 and 25-100 fmol of purified ER incrementally enhanced the protection of both the proximal and distal Sp1 sites (Lanes 4-6). As suggested from the gel mobility shift assays, the consensus ERE half site was protected in the presence of higher ER concentrations (Lane 6). When DNA fragments labeled with ^{32}P on the noncoding strand were incubated with 15 ng of purified Sp1 and increasing concentrations of purified ER, enhanced protection of both the proximal and distal Sp1 sites and the half ERE was observed (Lanes 9-12). As seen in the *in vitro* footprints with MCF-7 nuclear extracts and with purified Sp1, the proximal Sp1 site on the noncoding strand was more extensively protected than the distal Sp1 site. The ERE half site was partially protected on the noncoding strand. Control lanes containing DNA fragments, which had been exposed to DMS (Lanes 1 and 7) or DNase I (Lanes 2 and 8) in the absence of proteins, were included for reference.

Purified Sp1 and ER bind differentially to wt and mutant half ERE/Sp1 binding sites. The *in vitro* footprinting experiments reproducibly suggested a preference of Sp1 for the proximal Sp1 site. To determine how each of the Sp1 sites and the ERE half site contributed to protein/DNA complex formation, each of the individual elements was mutated and tested in gel mobility shift assays. Complementary oligos containing the wild type half ERE/Sp1 binding site (wt), or mutations in both Sp1 sites (mP/D), the distal Sp1 site (mD), the proximal Sp1 site (mP), or the ERE half site (mE) were synthesized, annealed, and labeled with ^{32}P. The labeled oligos were combined with purified Sp1 (Fig. 10, Lanes 1-5) or purified Sp1 and ER (Lanes 6-10) and fractionated on nondenaturing gels. Sp1 or Sp1 and ER bound effectively to the wt half ERE/Sp1 site (Lanes 1 and 6). As anticipated, Sp1 did not bind to the oligo containing mutations in both Sp1 sites, in the

absence (Lane 2) or in the presence of ER (Lane 7). Sp1 alone or in combination with ER bound to oligos containing a mutation in one of the two Sp1 binding sites, but more protein/DNA complex was formed when the oligo contained an intact proximal Sp1 site (mD; Compare Lanes 3 and 8 with Lanes 4 and 9). These findings corroborate the preferential binding of Sp1 to the proximal Sp1 site observed in the *in vitro* footprinting studies. The ability of ER to bind to oligos containing an intact ERE half site (Lanes 6-9), but not to an oligo containing a mutated ERE half site (Lane 10) further supports the idea that an ER monomer is bound to the ERE half site. When the ERE half site was mutated, increased Sp1/DNA complex formation was observed (Lanes 5 and 10). The reason for this apparent increase in Sp1 binding is unclear, but it was a reproducible finding.

Discussion

Sequence comparison of the PR gene from different species has been used to identify cis elements that are involved in estrogen-regulated transactivation. The rabbit PR gene contains an imperfect ERE, which overlaps with the translation start site and is capable of conferring estrogen responsiveness to a heterologous promoter in transient transfection assays (Savouret et al. 1991). Although a similar sequence is present in the chicken PR gene (Gronemeyer et al. 1987), no homologous sequence has been identified in the human PR gene (Kastner et al. 1990). A number of studies have suggested that ER and Sp1 may be involved in conferring estrogen responsiveness to the creatine kinase B (Wu-Peng et al. 1992), c-myc (Dubik and Shiu 1992), retinoic acid receptor α (Rishi et al. 1995), heat shock protein 27 (Porter et al. 1997; Porter et al. 1996), cathepsin D (Krishnan et al. 1994), and uteroglobin (Scholz et al. 1998) genes. The identification of an ERE half site adjacent to two Sp1 sites in the human PR gene (Kastner et al. 1990) led us to

investigate whether this region might be involved in conferring estrogen-responsiveness to the human PR gene. We initiated our studies by examining the endogenous PR gene in MCF-7 cells. Unlike transient transfection assays, which examine the ability of ER to activate transcription of synthetic promoters in supercoiled plasmids, our *in vivo* DNase I footprinting experiments allowed us to examine the endogenous PR gene as it exists in native chromatin and assess whether the half ERE/Sp1 binding site might play a physiological role in gene expression. E_2 treatment of MCF-7 cells did elicit more extensive protection of the half ERE/Sp1 binding site than was observed in the absence of hormone. The enhanced protection of the half ERE/Sp1 binding site seen after 72 hours of hormone treatment, a time when PR mRNA and protein reach maximal levels (Eckert and Katzenellenbogen 1982; Graham et al. 1996; Nardulli et al. 1988; Read et al. 1988; Wei et al. 1988), suggests that sustained protein-DNA interactions are required for maximal production of PR mRNA and protein. Furthermore, the ability of the half ERE/Sp1 binding site to enhance transcription of a CAT reporter plasmid in the presence of E_2 suggests that this region is involved in estrogen-responsiveness of the PR A promoter.

A role for Sp1 in regulating expression of the PR gene

Sp1 was originally described as a trans acting factor that bound to a GC box (5'GGGCGG3') and activated transcription of the SV40 promoter (Dynan and Tjian 1983; Gidoni et al. 1984). Subsequent comparison of numerous Sp1 binding sites led to the identification of a higher affintiy, consensus Sp1 site, 5'GGGGCGGGGC3' (Briggs et al. 1986) and the discovery that sequences, which varied from this consensus sequence, displayed decreased affinities for Sp1. While both of the Sp1 sites in the human PR half ERE/Sp1 binding site contain the GC box motif, only the proximal Sp1 site contains the 10 bp consensus Sp1 sequence (Fig. 1). The increased

affinity of Sp1 for the 10 bp proximal Sp1 site, when compared to the distal Sp1 site, was repeatedly observed in our *in vitro* footprinting assays and was most obvious on the noncoding strand (Figs. 5, 7, and 9). Gel mobility shift assays carried out with oligos containing mutations in the proximal or distal Sp1 site confirmed Sp1's preference for the proximal Sp1 site (Fig. 10). Interestingly, the centers of the two GC boxes present in the half ERE/Sp1 binding site are separated by 10 basepairs or one turn of the DNA helix ($Sp1_D$ +580 to +585, $Sp1_P$ +590 to +595). The periodicity of these elements could either favor interaction between adjacent DNA-bound proteins resulting in cooperative binding or stericly hinder binding of two Sp1 proteins. Our gel mobility shift and *in vitro* DNase I footprinting assays provided evidence for additive, not cooperative, binding of Sp1 to these sites and indicate that Sp1 binds first to the proximal Sp1 site and then to the distal Sp1 site.

A role for ER in regulating expression of the PR gene

The Sp1 sites in the endogenous PR A promoter were more protected after treatment of MCF-7 cells with E_2 in our *in vivo* footprinting experiments and E_2-occupied, but not unoccupied ER, effectively enhanced Sp1 binding to the two Sp1 sites in the PR A promoter in our *in vitro* binding assays. These findings suggest that an E_2-induced change in receptor conformation may be required for ER-enhanced Sp1 binding. Although we and others have been unable to detect ER interaction with the DNA-bound Sp1 in gel mobility shift assays, direct ER-Sp1 interaction has been documented in immunoprecipitation and GST pulldown experiments (Porter et al. 1997; Wang et al. 1998). This ability of the E_2-occupied ER to enhance Sp1 binding to DNA provides a mechanism by which estrogen could regulate genes that contain Sp1 sites.

Another way that estrogen might affect PR gene expression is through direct binding of the

receptor to the ERE half site. The ERE was protected in our *in vitro* footprinting experiments with ER and Sp1, but not with Sp1 alone demonstrating that the ER did bind to the ERE half site. Likewise, gel mobility shift experiments carried out with purified ER alone or in combination with Sp1 indicated that the ER bound surprisingly well to the ERE half site and formed a stable protein-DNA complex that was capable of withstanding the extensive periods of electorphoresis required for gel mobility shift experiments. Furthermore, the ERE was protected in our *in vivo* footprinting experiments after treatment of MCF-7 cells with E_2 suggesting that the ERE is involved in regulation of the endogenous gene. Although we were unable to detect protection of the ERE half site in our *in vitro* binding assays using MCF-7 nuclear extracts, the level of ER in these extracts (0.42 fmoles/μg protein) was significantly lower than the level present in an intact cell nucleus. Assuming a nuclear radius of 6 μm and 150,000 ER sites per cell (Clarke et al. 1989), the ER concentration in an MCF-7 nucleus would be 273 nM. These ER concentrations are significantly higher than the 0.25 - 2 nM concentrations used in our *in vitro* binding assays and would most likely favor ER binding to the ERE half site. The 10 bp separating the ERE half site and the distal Sp1 binding site would place the ER on the same side of the DNA helix as the DNA-bound Sp1 proteins and could help to foster protein-protein interactions.

We have considered only ERα in our studies since MCF-7 cells express high levels of ERα, but do not express ERβ (Clarke et al. 1989; Dotzlaw et al. 1996). Although we have not ruled out the possibility that another nuclear protein might bind to the ERE half site, the high levels of nuclear ER, the differential protection of the ERE half site in the presence and absence of E_2, and the demonstrated ability of ER to bind to the ERE half site *in vitro* suggest that it is most likely the ER that interacts with this site *in vivo* and helps to regulate transcription of the PR A promoter.

Regulation of the PR A Promoter in MCF-7 Cells

Our studies support the idea that ER and Sp1 are involved in estrogen-regulated expression of the human PR A promoter. The protection of nucleotides flanking the half ERE/Sp1 binding site in our *in vivo* footprinting experiment suggests that other proteins are associated with the promoter and are involved in transcription activation. Interestingly, the E_2-occupied ER, but not the unoccupied ER, interacts with a number of coactivator proteins, which participate in transcription activation and chromatin remodeling (Anzick et al. 1997; Hamstein et al. 1996; Hong et al. 1996; Norris et al. 1998; Ogryzko et al. 1996; Oñate et al. 1995; Smith et al. 1996; Thenot et al. 1997; Torchia et al. 1997). The recruitment of these proteins to the DNA-bound, liganded receptor could account for protection of sequences flanking the half ERE/Sp1 binding site and serve as the initiating event in the formation of an active transcription complex.

While models of DNA are typically drawn in a linear array, the packaging of DNA and protein into the nucleus of a cell requires tremendous compaction. This compaction could facilitate interaction between trans acting factors bound to more distant cis elements. Thus, the association of upstream activators such as ER and Sp1 with factors bound to downstream elements could be fostered. In fact, both ER and Sp1 are known to directly associate with TFIID components. ER interacts with TBP, TFIIB, and $TAF_{II}30$ (Ing et al. 1992; Jacq et al. 1994; Sabbah et al. 1998) and Sp1 interacts with TBP, $TAF_{II}130$, and $TAF_{II}55$ (Chiang and Roeder 1995; Emili et al. 1994; Gill et al. 1994; Tanese et al. 1996). The interaction of ER and Sp1 with TBP and its associated proteins could foster formation of a protein-protein network that helps to establish an active transcription complex. Furthermore, the E_2-dependent recruitment of coactivators such as CBP/p300, which can function as a histone acetyltransferase (Ogryzko et al.

1996), could help remodel chromatin in the PR A promoter and enhance formation of an interconnected protein-protein and protein-DNA network involved in activation of the human PR gene.

Figure Legends

Figure 1. Sequence of the half ERE/Sp1 binding site. The sequence of the half ERE/Sp1 binding site in the PR A promoter originally reported by Kastner et al (Kastner et al. 1990) is shown.

Figure 2. *In vivo* DNase I footprinting of the endogenous PR gene in MCF-7 cells. MCF-7 cells were maintained in serum-free medium for five days, treated with ethanol control (0 h E_2) or 1 nM E_2 for 2 or 72 hours, and then exposed to DNase I. Genomic DNA was isolated and used in *in vivo* LMPCR footprinting. Naked genomic DNA, which had been treated *in vitro* with either DMS (G) or DNase I (V_t), were included as references. The locations of the proximal Sp1 site ($Sp1_P$), distal Sp1 site ($Sp1_D$) and ERE half site are indicated.

Figure 3. Estrogen-enhanced activity of a plasmid containing the half ERE/Sp1 binding site. CHO cells were transfected with TATA CAT or ERE/Sp1-TATA CAT reporter plasmid, hER expression plasmid, β-galactosidase expression plasmid and pTZ nonspecific DNA using the calcium phosphate coprecipitation method as described in Experimental Procedures. Cells were treated with ethanol vehicle or 10 nM E_2. Data represent the average of 9 independent experiments. Values are presented as the mean $\pm$ SEM.

Figure 4. Binding of MCF-7 Sp1 protein to the half ERE/Sp1 site. ^{32}P-labeled oligos containing the half ERE/Sp1 binding site were incubated with nuclear extracts from E_2-treated MCF-7 cells. The ER-specific antibody H222 (ER Ab) or the Sp1 specific antibody IC6 (Sp1 Ab) was added to the binding reaction as indicated. The ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography.

Figure 5. *In vitro* footprinting of the half ERE/Sp1 binding site with MCF-7 nuclear extracts. 181bp DNA fragments containing the half ERE/Sp1 binding site were end-labeled on either the coding and noncoding strand and incubated with increasing concentrations of nuclear extract from E_2-treated MCF-7 cells (Lanes 3-5 and 8-10). The binding reactions were subjected to limited DNase I digestion and the cleaved DNA fragments were fractionated on a denaturing gel. Naked genomic DNA samples, which had been treated *in vitro* with either DMS (Lanes 1 and 6) or DNase I (Lanes 2 and 7), were included as references. The locations of the proximal Sp1 site ($Sp1_P$), distal Sp1 site ($Sp1_D$) and ERE half site are indicated.

Figure 6. Gel mobility shift assay of half ERE/Sp1 binding site-containing oligos and purified Sp1 protein. ^{32}P-labeled oligos containing the half ERE/Sp1 binding site were incubated with increasing concentrations of purified Sp1 protein and fractionated on a nondenaturing gel. The locations of the more rapidly (← 1) and more slowly (← 2) migrating Sp1/DNA complexes are indicated. The complexed and free DNA were visualized by autoradiography.

Figure 7. *In vitro* footprinting of the half ERE/Sp1 binding site with purified Sp1. 181bp DNA fragments containing the half ERE/Sp1 binding site and flanking regions were end-labeled on either the coding and noncoding strands and incubated with increasing concentrations of purified Sp1 protein (Lanes 3-5 and 8-10). The binding reactions were subjected to limited DNase I digestion and the cleaved DNA fragments were fractionated on a denaturing gel. Naked genomic DNA samples, which had been treated *in vitro* with either DMS (Lanes 1 and 6) or DNase I (Lanes 2 and 7), were included as references. The locations of the proximal Sp1 site ($Sp1_P$), distal Sp1 site ($Sp1_D$) and ERE half site are indicated.

Figure 8. ER-enhanced binding of Sp1 to the half ERE/Sp1 binding site. (Panel A) ^{32}P-labeled oligos containing the half ERE/Sp1 binding site were incubated with 3ng (Lane 1) or 0.25ng of purified Sp1(Lanes 2-6) and 5,10, 20 or 40 fmoles of purified ER (Lane 3-6). ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography. (Panel B) ^{32}P-labeled oligos containing the half ERE/Sp1 binding site were incubated with purified Sp1 (Lanes 1-3), purified ER (Lanes 4-6) or purified Sp1 and ER (Lanes 7-9). The ER-specific antibody H222 (ER Ab) or the Sp1 specific antibody IC6 (Sp1 Ab) were added to the binding reaction as indicated. ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography.

Figure 9. *In vitro* DNase I footprinting of the half ERE/Sp1 binding site with purified Sp1 and ER. DNA fragments containing the half ERE/Sp1 binding site and flanking regions, were end-labeled on either the coding and noncoding strands and incubated with 15ng of purified Sp1 protein (Lanes 3-6 and 9-12) and 25, 50 or 100 fmoles of purified ER (Lanes 4-6 and 10-12). The binding

reactions were subjected to limited DNase I digestion and the cleaved DNA fragments were fractionated on a denaturing gel. Naked genomic DNA samples, which had been treated *in vitro* with either DMS (Lanes 1 and 7) or DNase I (Lanes 2 and 8), were included as references. The locations of the proximal Sp1 site ($Sp1_P$), distal Sp1 site ($Sp1_D$) and ERE half site are indicated.

Figure 10. Interaction of purified Sp1 and ER with wild type and mutant half ERE/Sp1 binding sites. ^{32}P-labeled oligos containing the wild type half ERE/Sp1 binding site (wt), or mutations in both Sp1 binding sites (mP/D), the distal Sp1 binding site (mD), the proximal Sp1 binding site (mP), or the ERE half site (mE) were incubated with 3ng of purified Sp1 (Lanes 1-5) or 3ng of purified Sp1 and 10 fmoles of purified ER (Lane 6-10). The ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography.

KEY RESEARCH ACCOMPLISHMENTS

- In vivo DNase I footprinting demonstrates that the half ERE/Sp1 binding site is more protected when MCF-7 cells are treated with estrogen than when cells are not exposed to hormone.

- *In vitro* DNase I footprinting and gel mobility shift assays demonstrate that Sp1 present in MCF-7 nuclear extracts and purified Sp1 protein bind preferentially to the proximal Sp1 site and then to the distal Sp1 site.

- Purified estrogen-occupied receptor enhances Sp1 binding and binds directly to the ERE half site.

- The half ERE/Sp1 binding site can confer estrogen responsiveness to a heterologous reporter plasmid.

- Our studies support the idea that ER and Sp1 are involved in estrogen-regulated expression of the human PR A promoter.

REPORTABLE OUTCOMES

Abstracts

Petz LN and Nardulli, AM (1999) Regulation of the Progesterone Receptor Gene by Estrogen in MCF-7 Human Breast Cancer Cells, Proceeding, 81st Annual Endocrine Society, P1-270, p.192

Petz LN and Nardulli AM (2000) Regulation of the Progesterone Receptor Gene, Breast Cancer Research Program

Manuscripts

Sp1 Binding Sites and An Estrogen Response Element Half Site Are Involved in Regulation of the Progesterone Receptor A Promoter, Submitted.

Presentations

Sixth Biennial Retreat in Reproductive Biology, Allerton Conference Center, Monticello, IL, June 1999

University of West Virginia, Department of Pharmacology and Toxicology, October 1999

University of Louisville School of Medicine, Department of Biochemistry and Molecular Biology, November, 1999

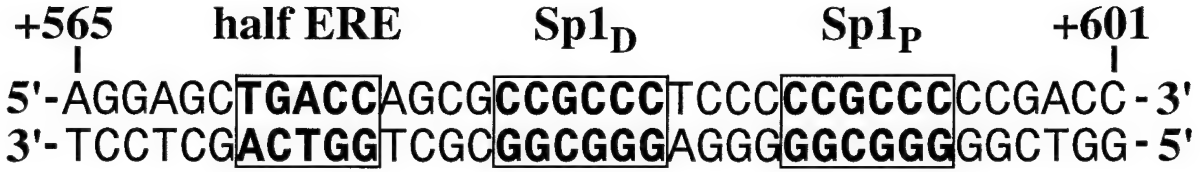
This work and studies supported by another DOD grant will form the basis of a new NIH grant proposal on mechanisms involved in regulation of estrogen-responsive genes.

CONCLUSIONS

The overall goal of this study was to better understand how the PR gene is regulated in human breast cancer cells. Ours is the first study to define cis elements and trans acting factors involved in regulation of the human progesterone receptor gene. Our findings provide novel information about the involvement of Sp1 and the estrogen receptor in regulating PR expression in human breast cancer cells suggest that these proteins may influence growth of mammary cells by regulating the level of PR.

LIST OF PERSONNEL SUPPORTED BY THIS GRANT

Dr. Larry Petz
Dr. Jongsook Kim
Margaret Grim Loven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Sp1P
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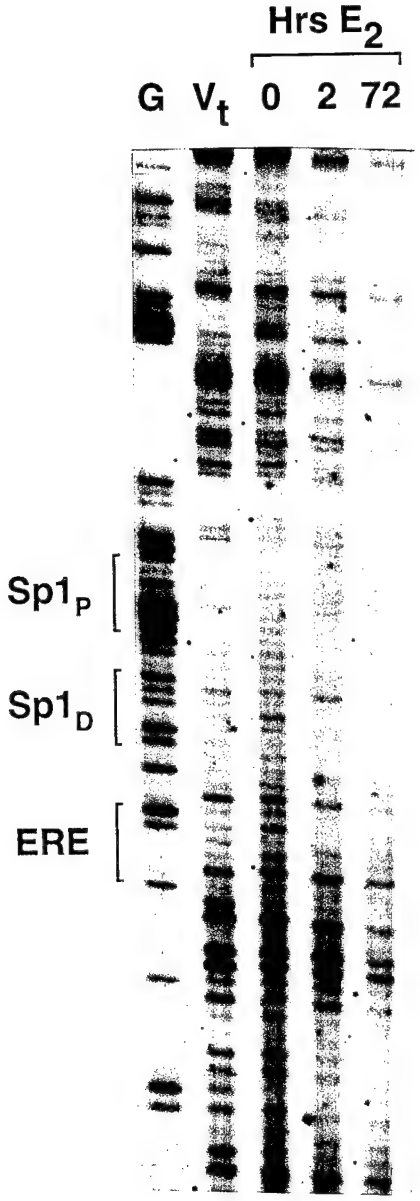
Figure 1



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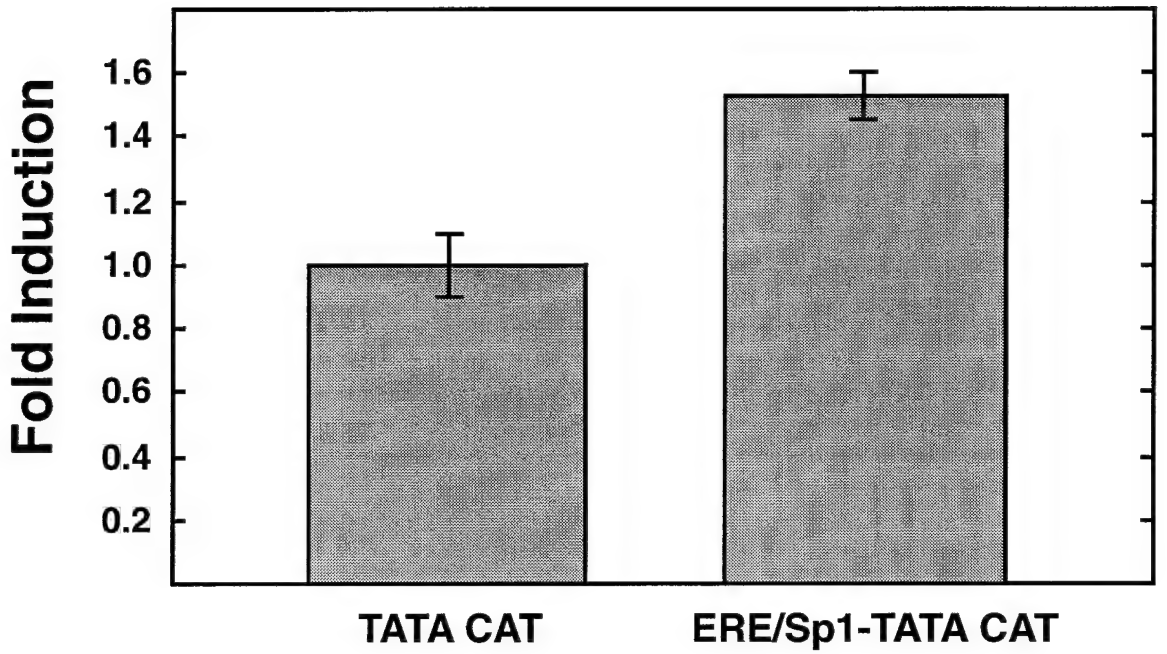

Figure 3

Figure 4

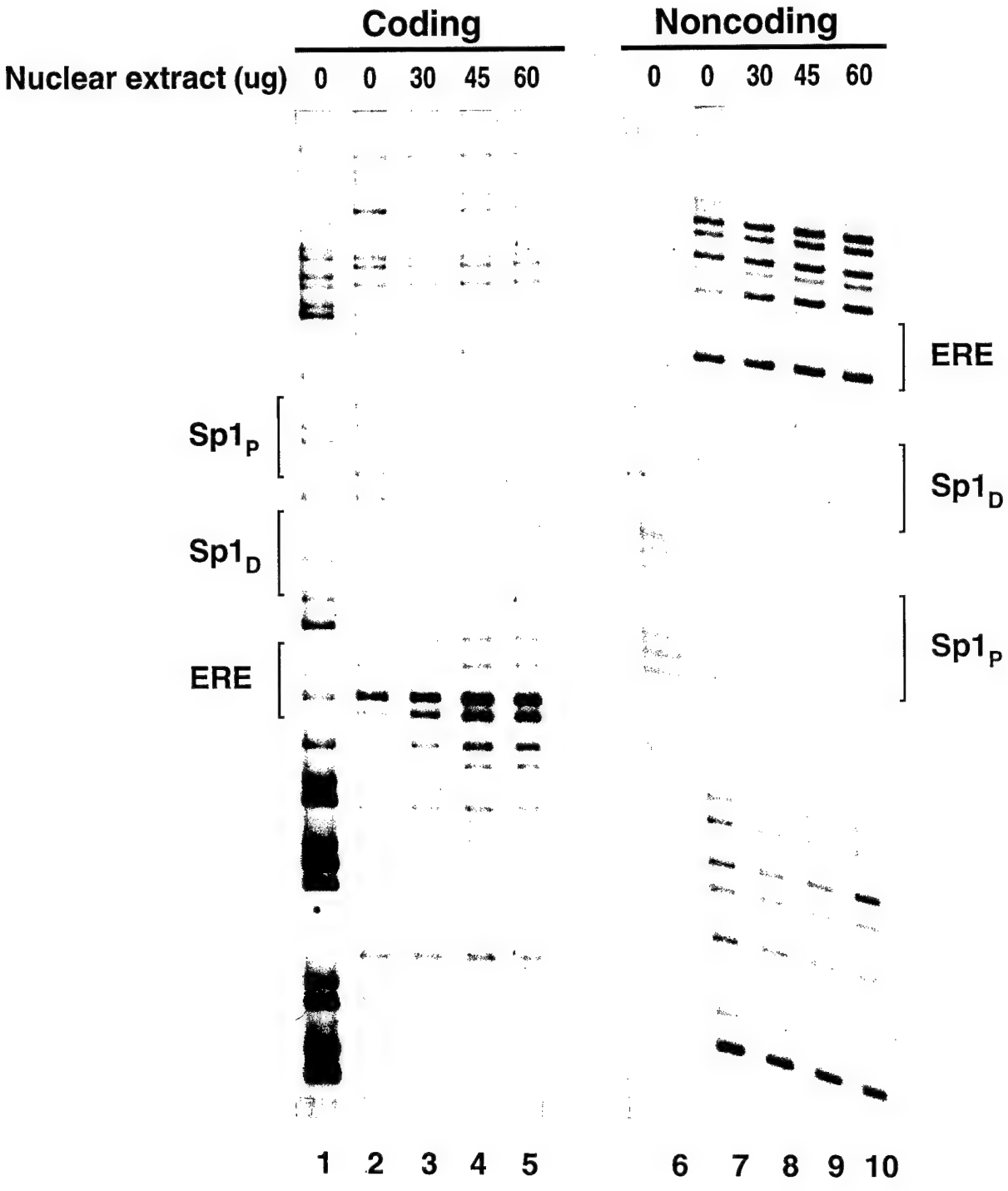
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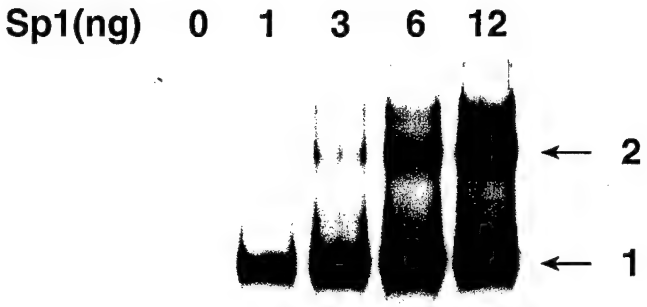
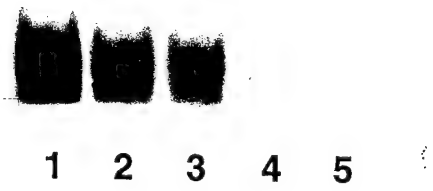
Figure 5





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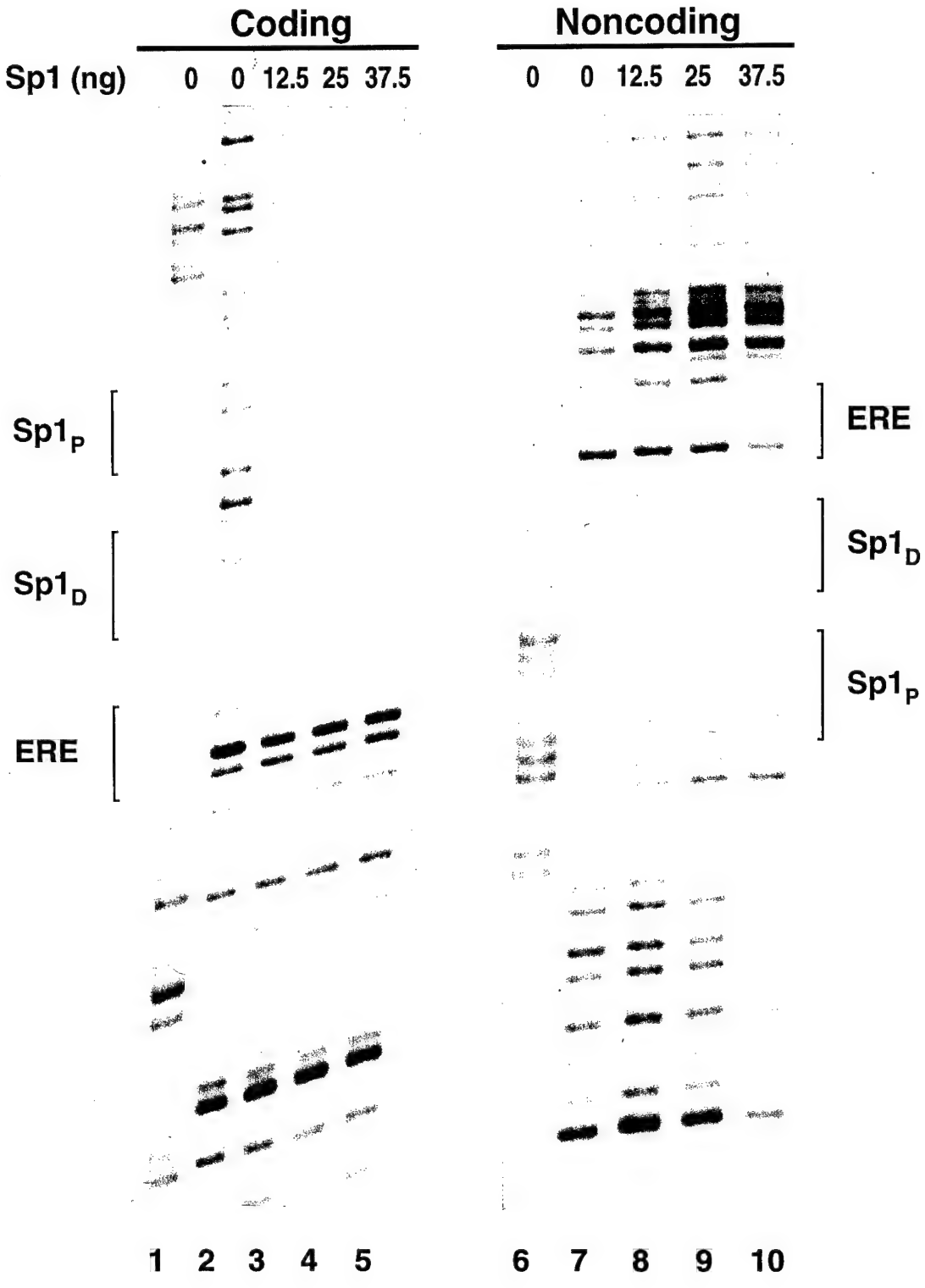
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Figure 7

A

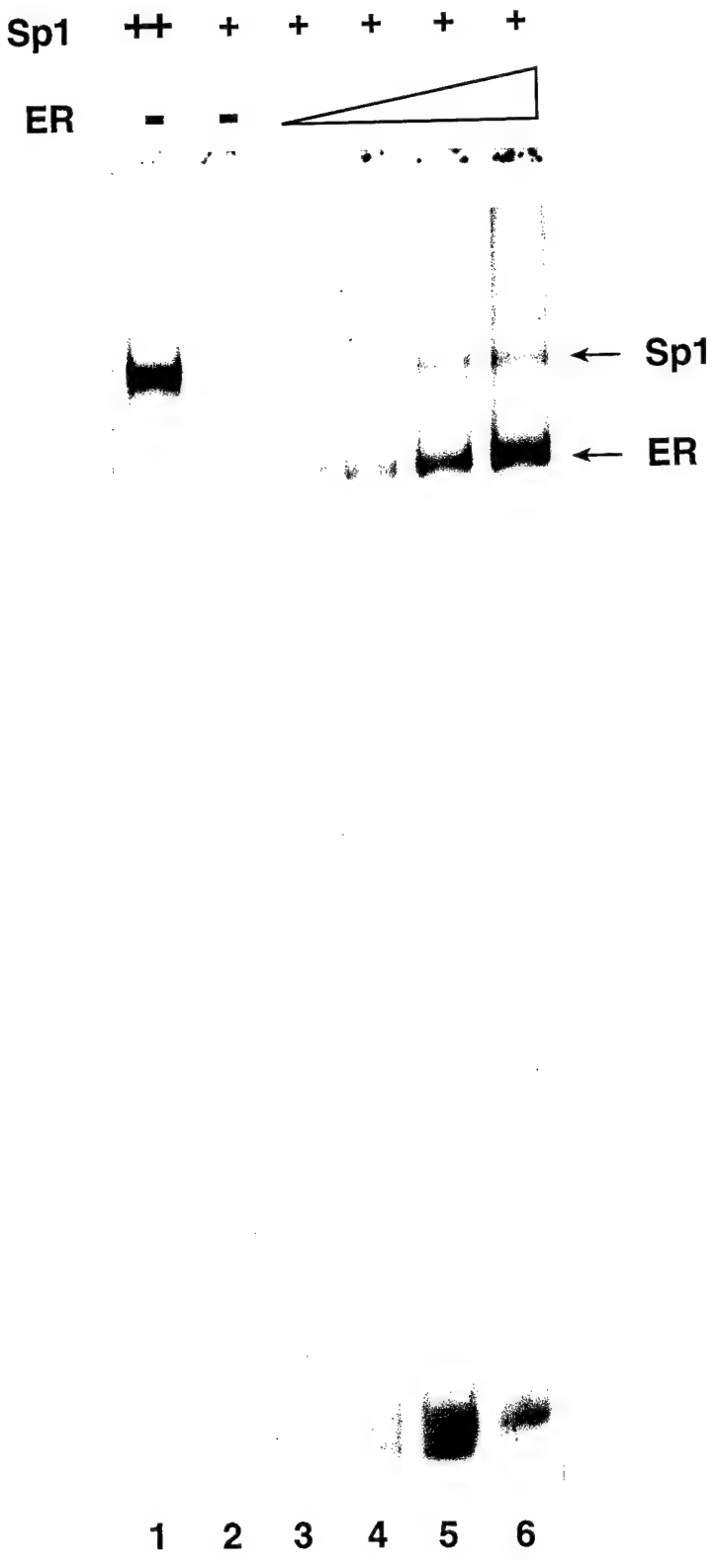
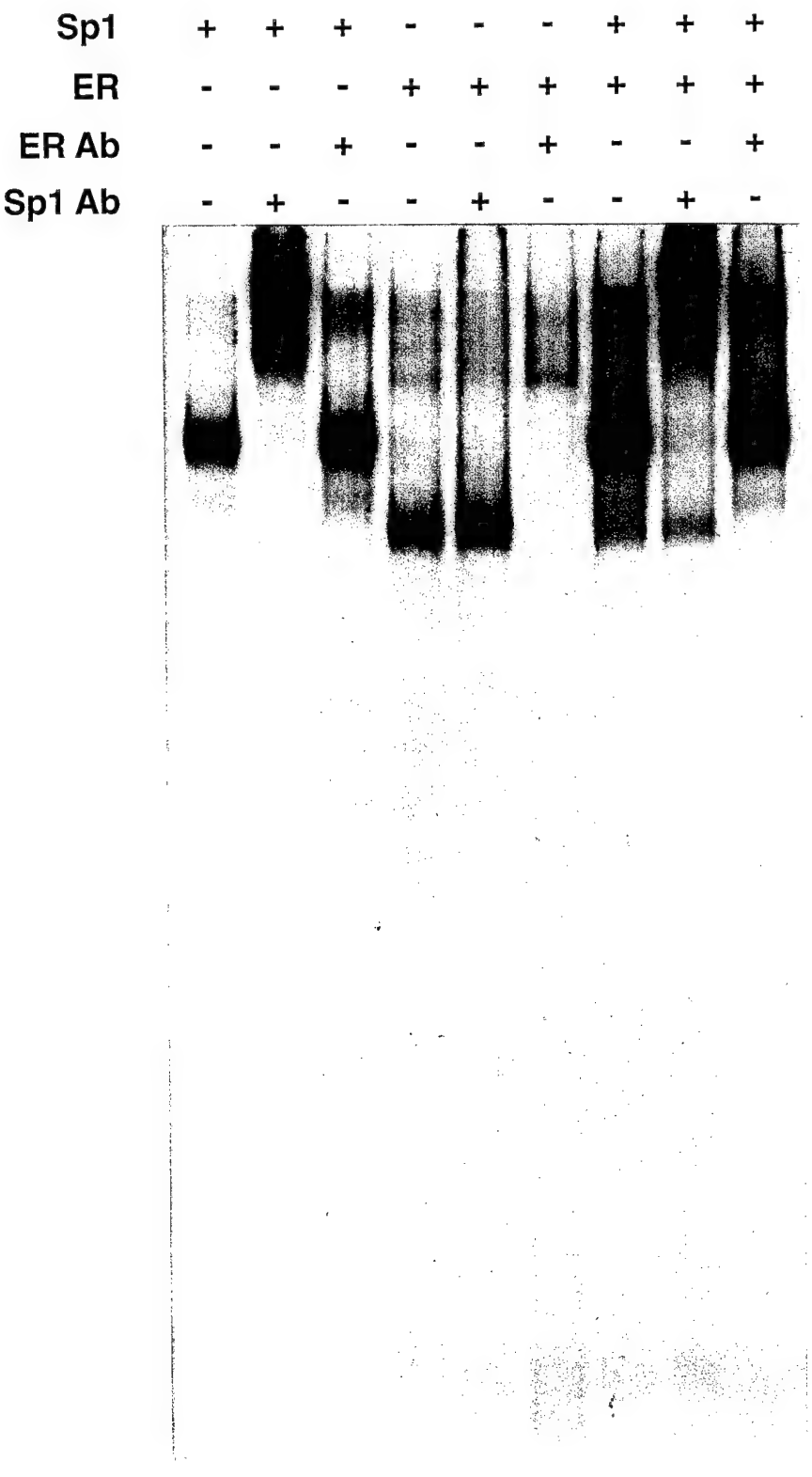


Figure 8A

B



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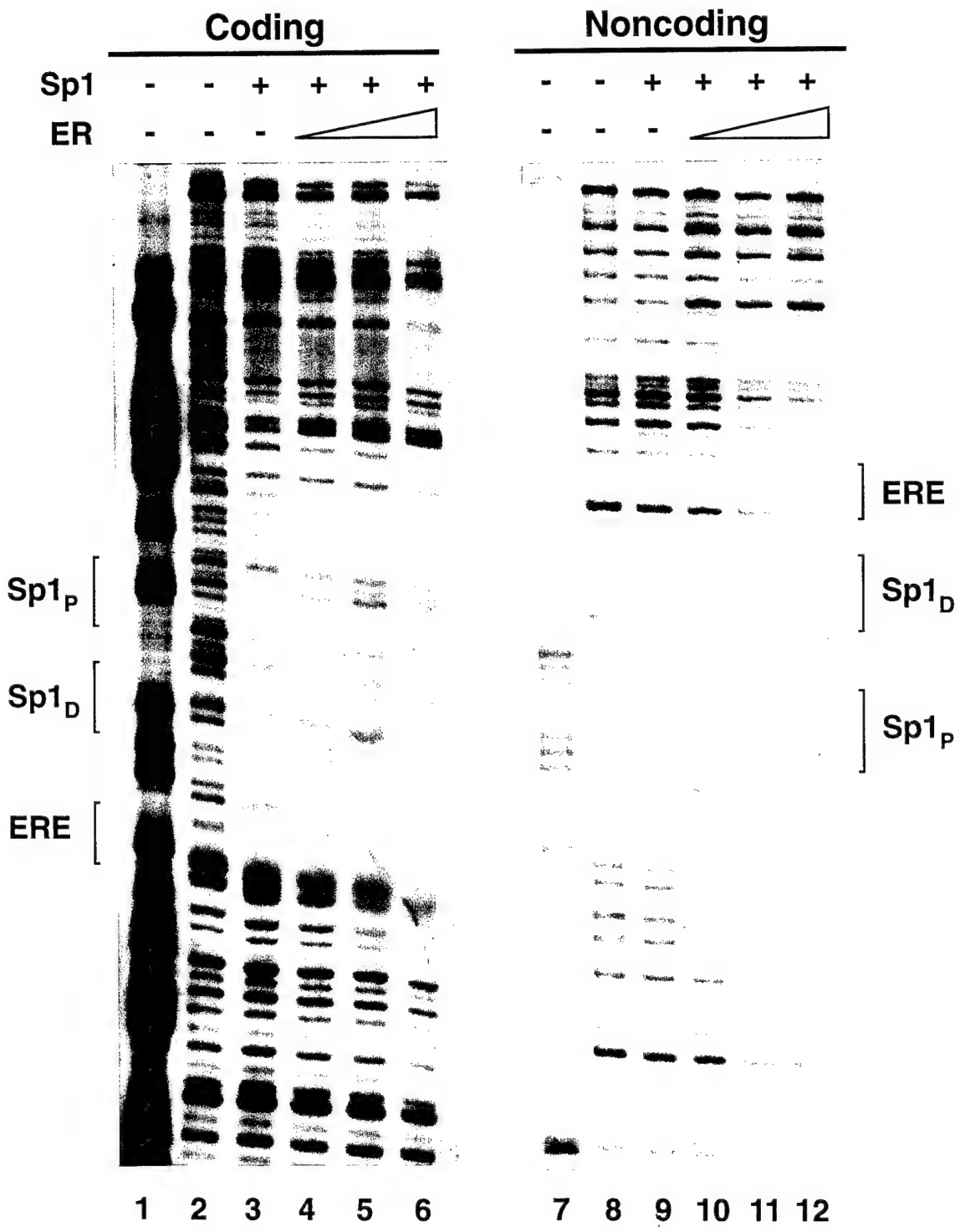
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Figure 9

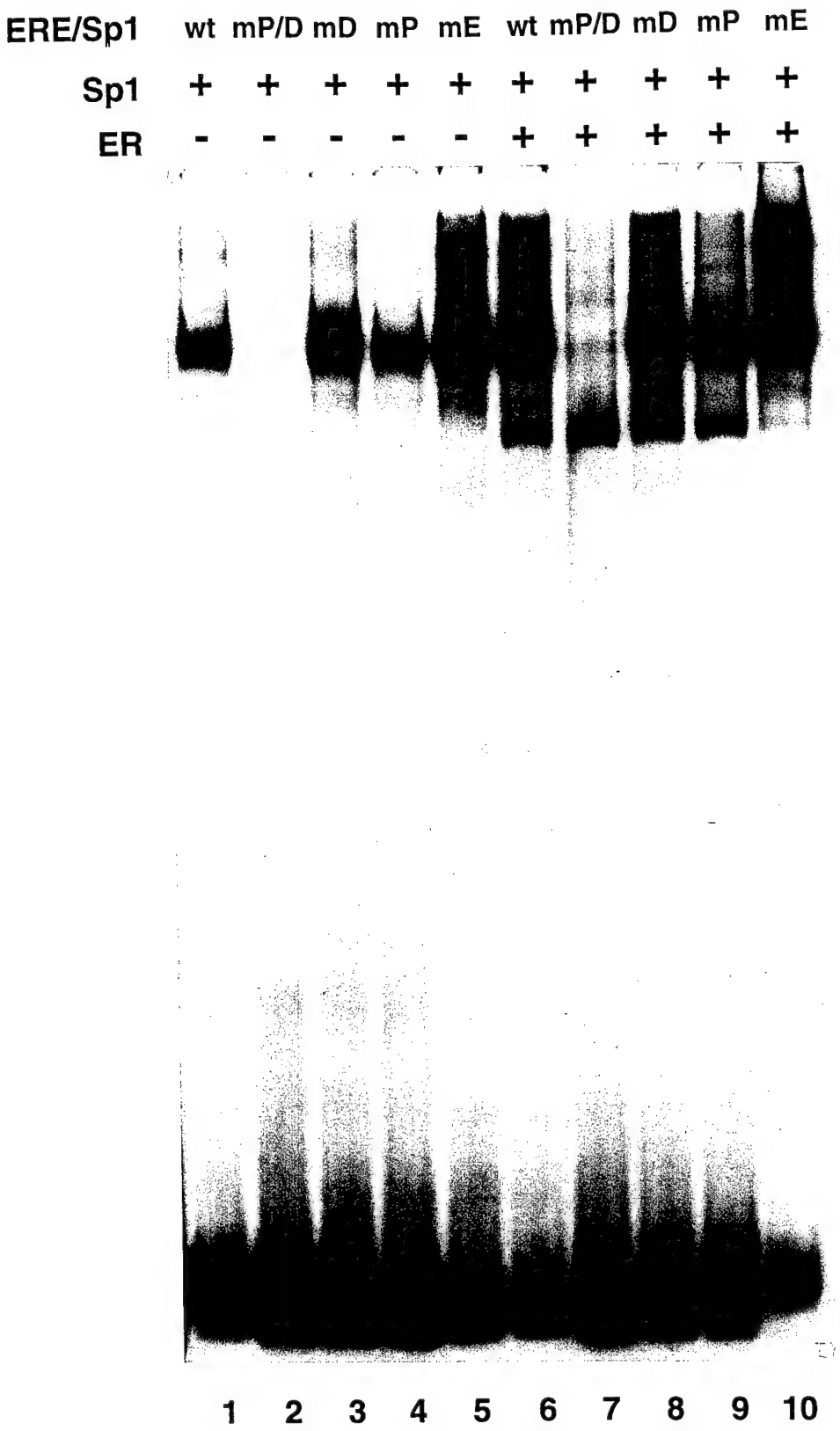

Figure 10

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Sp1 Binding Sites and An Estrogen Response Element Half Site Are Involved in Regulation of the Human Progesterone Receptor A Promoter

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Summary

Progesterone receptor gene expression is induced by estrogen in MCF-7 human breast cancer cells. Although it is generally thought that estrogen-responsiveness is mediated through estrogen response elements (EREs), the progesterone receptor gene lacks an identifiable ERE. The progesterone receptor A promoter does, however, contain a half ERE/Sp1 binding site comprised of an ERE half site upstream of two Sp1 binding sites. We have used *in vivo* DNase I footprinting to demonstrate that the half ERE/Sp1 binding site is more protected when MCF-7 cells are treated with estrogen than when cells are not exposed to hormone suggesting that the this region is involved in estrogen-regulated gene expression. The ability of the half ERE/Sp1 binding site to confer estrogen responsiveness to a simple heterologous promoter was confirmed in transient cotransfection assays. *In vitro* DNase I footprinting and gel mobility shift assays demonstrated that Sp1 present in MCF-7 nuclear extracts and purified Sp1 protein bound to the two Sp1 sites and that estrogen-occupied estrogen receptor enhanced Sp1 binding. In addition to its effects on Sp1 binding, the estrogen receptor also bound directly to the ERE half site. Taken together, these findings suggest that estrogen-occupied receptor and Sp1 play a role in activation of the human progesterone receptor A promoter.

Introduction

Estrogen is a hormone of critical importance in the development and maintenance of reproductive tissues and also plays an important role in cardiovascular and bone physiology. Estrogen's effects are mediated through its interaction with the intracellular estrogen receptor (ER). Numerous studies have demonstrated that the two ERs, α and β, mediate their effects by binding to specific DNA sequences, estrogen responsive elements (EREs), thereby initiating changes in transcription of target genes (1,2).

It has become apparent that, in addition to binding directly to an ERE, the ER may also modulate transcription indirectly by interacting with other DNA-bound proteins. For example, ER interaction with AP1-bound fos and jun proteins confer estrogen responsiveness to the ovalbumin (3), c-*fos* (4), collagenase (5), and insulin-like growth factor I (6) genes. In addition, a growing body of evidence suggests that the ER may influence binding of Sp1 to its recognition site and thereby confer estrogen responsiveness to the creatine kinase B (7), c-myc (8), retinoic acid receptor α (9), heat shock protein 27 (10,11), cathepsin D genes (12), and uteroglobin (13) genes.

The progesterone receptor (PR) gene is under estrogen control in normal reproductive tissues (14,15) and in MCF-7 human breast cancer cells (16-18). MCF-7 PR mRNA and protein increase and reach maximal levels after three days of 17 β-estradiol (E_2) treatment (16-18). Like ER, two distinct PR forms are differentially expressed in a tissue-specific manner (19-23). PR-B is a 120 kD protein containing a 164 amino acid amino-terminal region that is not present in the 94 kD PR-A. Two discrete promoters, A and B, which are responsible for the production of PR-A and PR-B, respectively, have also been defined (24). The activities of these two promoters are

increased by estrogen treatment of transiently transfected Hela cells. Interestingly, no consensus EREs have been identified in either Promoter A (+464 to 1105) or Promoter B (-711 to +31). Promoter A does, however, contain an ERE half site located upstream of two Sp1 sites (24). The presence of these adjacent binding sites suggests that the ER might be able to influence PR expression directly by binding to the ERE half site, indirectly by interacting with proteins bound to the putative Sp1 sites, or a combination of these two methods. To determine whether the ERE half site and the two Sp1 sites present in the human PR A promoter might impart estrogen responsiveness to the PR gene, a series of *in vivo* and *in vitro* experiments were carried out.

Experimental Procedures

Cell Culture. MCF-7 human breast cancer cells (25) were maintained in Eagle's Minimum Essential Medium (MEM) containing 5% heat-inactivated calf serum. Cells were seeded in 10 cm plates and transferred to phenol red free, serum free Improved MEM (26) five days before the experiments were conducted. Chinese Hamster Ovary (CHO) cells were maintained in DMEM/F12 supplemented with 5% charcoal dextran stripped calf serum (27).

Oligonucleotides and Plasmid Constructions. The names and sequences of wildtype (wt) or mutant half ERE/Sp1 binding site are listed. Nucleotides that differ from the endogenous, wt half ERE/Sp1 binding site are underlined.

ERE/Sp1 wt: 5'-GATCTAGGAGCTGACCAGCGCCGCCCTCCCCCGCCCCCGACCA-3'

and 5'-GATCTGGTCGGGGGCGGGGGAGGGCGGCGCTGGTCAGCTCCTA-3',

ERE/Sp1 mP/D: 5'-GATCTAGGAGCTGACCAGCGTTGTACTCCCTTGTACCCGACCA-3'

and 5'-GATCTGGTCGGGTACAAGGGAGTACAACGCTGGTCAGCTCCTA-3',

ERE/Sp1 mD: 5'-GATCTAGGAGCTGACCAGCGTTGTACTCCCCCGCCCCCGACCA-3'

and 5'-GATCTGGTCGGGGGCGGGGGAGTACAACGCTGGTCAGCTCCTA-3',

ERE/Sp1 mP: 5'-GATCTAGGAGCTGACCAGCGCCGCCCTCCCTTGTACCCGACCA-3'

and 5'-GATCTGGTCGGGTACAAGGGAGGGCGGCGCTGGTCAGCTCCTA-3',

ERE/Sp1 mE: 5'-GATCTAGGAGCTGATTAGCGCCGCCCTCCCCCGCCCCCGACCA-3'

and 5'-GATCTGGTCGGGGGCGGGGGAGGGCGGCGCTAATCAGCTCCTA-3'.

ERE/Sp1 wt oligos with Bgl II compatible ends were subcloned into the *Bgl* II-cut, dephosphorylated chloramphenicol acetyl transferase (CAT) reporter plasmid, TATA CAT (28),

to create ERE/Sp1-TATA CAT. The ligated vector was transformed into the DH5α strain of *E. coli*, sequenced, and purified on two cesium chloride gradients.

In vitro and *in vivo* treatment of genomic DNA. MCF-7 cells were exposed to ethanol vehicle or 1 nM E_2 for 0, 2, or 72 hours prior to DNase I treatment. Cells were permeabilized with 0.4% NP-40 and treated with 750 U DNase I / ml (Boehringer Mannheim, Indianapolis, IN) for 3 min at 25°C. Isolation of genomic DNA was carried out as described by Mueller and Wold (29). The genomic DNA was purified, incubated with RNase A, resuspended in TE (10mM Tris pH 7.5, 1mM EDTA) and stored at -20 °C.

Naked genomic DNA was treated *in vitro* with dimethylsulfate (DMS) as described (29). *In vitro* DNase I-treated DNA was prepared by adjusting 100 μg of protein-free, RNase A-treated DNA to 175 μl with TE. DNA was incubated with 2.5 x 10^{-5} U DNase I for 5 min at 37° C. The reaction was stopped by the addition of 10 mM EDTA and processed as described for *in vivo*-treated genomic DNA.

In vivo footprinting. Ligation mediated PCR (LMPCR) footprinting was carried out essentially as described by Mueller and Wold (29,30). 2 μg of genomic DNA was subjected to LMPCR procedures using nested primers, which annealed to sequences upstream of the half ERE/Sp1 binding site (+571 to +595) in the human PR gene. The primer sequences were: Primer 1- 5'TCCCCGAGTTAGGAGACGAGAT3', Primer 2- 5'CGCTCCCCACTTGCCGCTC3', and Primer 3- 5'GCTCCCCACTTGCCGCTCGCTG3'. The annealing temperatures for the primers were 55°, 62°, and 69°, respectively. The linker primers LMPCR 1 and LMPCR 2 described by Mueller and Wold (30) were also used, except that LMPCR 1 was modified by removing the two 5' nucleotides to eliminate potential secondary structure.

In vitro DNase I footprinting. Primers, which annealed 88 bp upstream (Primer 3) or 79 bp downstream (Primer 4-5'TCGGGAATATAGGGGCAGAGGGAGGAGAA3') of the half ERE/Sp1 binding site, were subjected to 30 rounds of PCR amplification with 30 ng of the PR-(+464/+1105) CAT (24). Labeling of the coding and noncoding strands was carried out with ^{32}P-labeled Primer 3 or Primer 4, respectively. The 181 bp singly end-labeled amplified fragments were fractionated on an acrylamide gel and isolated. End-labeled DNA fragments (100,000 cpm) containing the half ERE/Sp1 binding site were incubated for 15 min at room temperature in a buffer containing 10% glycerol, 50 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, 1 mM $MgCl_2$, 50ng of poly dIdC and 0.4 mM DTT in a final volume of 50 µl with either 30-60 µg of MCF-7 nuclear extract, 12.5-37.5 ng of purified Sp1 protein (Promega, Madison, WI) or 15 ng of purified Sp1 and 25-100 fmol of purified Flag-tagged ER, which had been expressed and purified as described by Kraus and Kadonaga (31). 10 nM E_2 was included in binding reactions containing the purified ER. Bovine serum albumin (BSA) was included with the purified Sp1 protein or the purified Sp1 and ER so that the total protein concentration in each reaction was 25 µg. When MCF-7 nuclear extracts were used, ovalbumin and KCl were added as needed to maintain constant protein and salt concentrations and poly dI/dC was increased to 1 µg per reaction. 1 - 2 U of RQ1 ribonuclease-free DNase I (Promega, Madison, WI) was added to each sample and incubated at room temperature for 0.75- 8 min. The DNase I digestion was terminated by addition of stop solution (200mM NaCl, 1% SDS, 30 mM EDTA and 100 ng/µl tRNA) The DNA was phenol/chloroform extracted, precipitated, and resuspended in formamide loading buffer (32). Samples were fractionated on an 8% denaturing acrylamide gel. Radioactive bands were

visualized by autoradiography and quantitated with a Molecular Dynamics phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Gel mobility shift assays. Gel mobility shift assays were carried out essentially as described (33,34). ^{32}P-labeled (10,000 cpm) half ERE/Sp1-containing wild type or mutant oligos were incubated for 15 min. at room temperature in a buffer containing 10% glycerol, 50 mM KCl, 15 mM Tris, pH 7.9, 0.2 mM EDTA, 1 mM $MgCl_2$, 50ng of poly dI/dC and 0.4 mM DTT in a final volume of 20 µl with either 20 µg of MCF-7 nuclear extract, 0.25-3 ng of purified Sp1 protein, or 0.25 ng of purified Sp1 and 5-40 fmol of purified ER.10 nM E_2 was included in all binding reactions containing ER. BSA was included when purified Sp1 or ER were used so that the total protein concentration in each reaction was 20 µg. When MCF-7 nuclear extracts were used, the nonspecific DNA for each reaction included 1 µg of salmon sperm DNA and poly dI/dC was increased to 2 µg. For antibody supershift experiments, the Sp1-specific monoclonal antibody, 1C6 (Santa Cruz Biotech, Santa Cruz, CA) or the ER-specific monoclonal antibody, H222, (Kindly provided by Dr. Geoffrey Greene, University of Chicago, Chicago, IL) was added to the protein-DNA mixture and incubated for 10 min at room temperature. Low ionic strength gels and buffers were prepared as described (32). Radioactive bands were visualized by autoradiography.

Transient transfection of CHO cells. CHO cell transfections were performed using the calcium phosphate method (35). Crystals were formed in the presence of 3 µg of the indicated CAT reporter, 200 ng of the β-galactosidase vector pCH110 (Pharmacia, Piscataway, NJ), 5 ng of the human ERα expression vector pCMVhER (36), and 4.8 µg of pTZ18U and incubated with CHO cells for 16 hrs followed by a 2 min 20% glycerol shock. Cells were maintained in media containing ethanol vehicle or 10 nM E_2 for 24 hrs. Protein concentration was determined using

Bio-Rad (Hercules, PA) protein assay with BSA as a standard. Mixed-phase CAT assays were performed using 35 μg protein as previously described (37). The β-galactosidase activity was determined at room temperature as previously described (38) and used to normalize the amount of CAT activity in each sample.

Results

In vivo footprinting of the PR gene. A number of studies have suggested that an Sp1 site alone or in combination with an imperfect ERE or ERE half site may be involved in conferring estrogen responsiveness to target genes (7-13). To determine whether the ERE half site and two potential Sp1 sites residing in the endogenous human PR gene (+571 to +595, Ref. 24) might be involved in estrogen-regulated transactivation, *in vivo* DNase I footprinting was carried out using MCF-7 cells. The region of the PR A promoter containing the consensus ERE half site and two potential Sp1 sites is shown in Fig. 1 and will be hereafter referred to as the half ERE/Sp1 binding site.

To carry out the *in vivo* footprinting assays, MCF-7 cells were treated with ethanol vehicle or with E_2 for 2 or 72 hours and then exposed to DNase I. The cells were rapidly lysed, DNA was isolated, and LMPCR procedures were carried out (29). Naked genomic DNA, which had been treated *in vitro* with DNase I served as a reference in identifying sequences that were susceptible to cleavage in the absence of proteins (Fig. 2, V_t). When cells were treated with E_2 for 2 hours, the protection of the proximal Sp1 site ($Sp1_P$), the distal Sp1 binding site ($Sp1_D$), and the ERE half site was greater than seen in cells that had not been exposed to hormone. After 72 hours of E_2 treatment, a time when PR mRNA and protein reach maximal levels (16-18,27,39), the protection of the half ERE/Sp1 site was sustained. E_2 treatment also elicited protection of regions flanking the half ERE/Sp1 binding site. Thus, we were able to detect distinct differences in protection of the half ERE/Sp1binding site on the coding strand of the endogenous PR gene after E_2 treatment. Despite numerous attempts, we were unable to obtain a footprint of the noncoding PR DNA strand in this region. The failure of the LMPCR reactions may have been due to formation of an extensive stem loop structure (ΔG = - 11.5 Kcal/mol) extending from

+ 674 to + 733 (24) that limited primer annealing or interfered with the ability of polymerase to proceed through this region. None the less, our *in vivo* footprinting of the coding strand demonstrated that the half ERE/Sp1 binding site residing in the endogenous PR gene was differentially protected in ethanol- and E_2-treated MCF-7 cells and suggested that the ERE half site as well as the proximal and distal Sp1 sites might be involved in regulation of the endogenous PR gene in MCF-7 cells.

Estrogen enhances transcription of a reporter plasmid containing the half ERE/Sp1 binding site. To determine if the half ERE/Sp1 binding site could confer estrogen-responsiveness to a heterologous promoter, transient cotransfection experiments were carried out with a human ER expression vector and a CAT reporter plasmid containing either a TATA box (TATA CAT) or the half ERE/Sp1 binding site and a TATA box (ERE/Sp-TATA CAT). Exposure of transiently cotransfected CHO cells to E_2 resulted in an increase in CAT activity when the reporter plasmid contained the half ERE/Sp1 binding site (Fig. 3 ERE/Sp1-TATA CAT). In contrast, no change in activity was observed with E_2 treatment when the parental reporter plasmid containing a TATA box was used (TATA CAT). These findings suggest that the half ERE/Sp1 binding site is involved in estrogen-mediated activation of the PR A promoter.

Proteins present in MCF-7 nuclear extracts bind to the half ERE/Sp1 binding site *in vitro*. Our *in vivo* footprinting and transient transfection experiments provided evidence for the involvement of the half ERE/Sp1 binding site in mediating estrogen's effects on the PR A promoter. However, these studies did not allow us to identify proteins that interact with this DNA sequence. To begin to identify proteins that bind to this site, gel mobility shift assays were carried out with MCF-7 nuclear extracts. When ^{32}P-labeled oligos, each containing the half ERE/Sp1 binding site, were

combined with nuclear extracts prepared from E_2-treated MCF-7 cells, one major protein-DNA complex was formed (Fig. 4, Lane 1). Since we anticipated that ER and Sp1 might bind to this region, antibodies to these proteins were included in separate binding reactions. The major protein-DNA complex was supershifted by the Sp1-specific antibody 1C6, which binds only to Sp1 and does not cross react with Sp2-4 (Lane 2). In contrast, the major protein-DNA complex was not affected by the ER-specific antibody H222 (Lane 3). These data indicate that Sp1 was present in substantial amounts in our MCF-7 nuclear extracts and that it bound efficiently to the half ERE/Sp1 binding site. However, these experiments did not provide evidence that the ER was involved in formation of the protein-DNA complex.

Gel mobility shift experiments require the formation of stable protein-DNA complexes, which must be maintained during extended periods of electrophoresis. To determine whether more transient or lower affinity interaction might occur between MCF-7 nuclear proteins and the ERE half site and/or either one or both of the Sp1 binding sites, *in vitro* DNase I footprinting was carried out. 181bp DNA fragments, each containing the half ERE/Sp1 binding site and additional PR flanking sequence, were ^{32}P-labeled on one end, incubated with increasing amounts of MCF-7 nuclear extract, and exposed to limited DNase I cleavage (Fig. 5, Lanes 3-5 and 8-10). When DNA fragments, which had been ^{32}P -labeled on the coding strand were utilized, the proximal and distal Sp1 sites were partially protected by proteins present in the MCF-7 nuclear extracts (Lanes3-5). Quantitative analysis of the coding strand revealed slightly greater protection of the proximal Sp1 site than the distal Sp1 site. Although the ERE half site was not protected, nucleotides within and immediately flanking the ERE half site were hypersensitive to DNase I cleavage upon addition of increasing concentrations of nuclear proteins (Lanes 3-5). When the

noncoding DNA strand was labeled and utilized in *in vitro* footprinting experiments with MCF-7 nuclear extracts, the proximal Sp1 site was more extensively protected than the distal Sp1 site (Lane 8-10). As seen with the coding strand, hypersensitive sites were observed within and adjacent to the ERE half site on the noncoding strand. Control lanes containing DNA fragments, which had been exposed to DMS (Lanes 1 and 6) or DNase I (Lanes 2 and 7) in the absence of protein, were included for reference. The enhanced protection of the Sp1 sites observed in our *in vitro* footprints in the presence of MCF-7 nuclear extracts was similar to the increased protection of the Sp1 sites in the endogenous gene upon E_2 treatment of MCF-7 cells. The ERE half site was not protected in our *in vitro* footprints as seen in the *in vivo* footprints, but rather displayed hypersensitivity to DNase I cleavage on both strands. Since DNase I hypersensitivity can result from binding of a protein to the major groove of the DNA helix making the minor groove more accessible to DNase I cleavage (40), the hypersensitivity observed within and adjacent to the ERE could result from binding of a protein to the major groove in the region of the ERE.

Purified Sp1 binds to the half ERE/Sp1 binding site. Our antibody supershift experiments indicated that native Sp1 present in MCF-7 nuclear extracts was binding to the half ERE/Sp1 binding site. However, the MCF-7 extracts used in these assays contained a complex combination of nuclear proteins. To determine whether the Sp1 protein alone was capable of binding to the half ERE/Sp1 binding site or whether other proteins present in the MCF-7 nuclear extracts were required for Sp1 binding, gel mobility shift experiments were carried out with purified Sp1 protein. ^{32}P-labeled oligos, each containing the half ERE/Sp1 binding site, were incubated with increasing concentrations of purified Sp1 protein and fractionated on a nondenaturing acrylamide gel (Fig. 6, Lanes 2-5). At the lowest Sp1 concentration utilized (1 ng), a single gel-shifted band

was observed (←1, Lane 2). As increasing concentrations of Sp1 were added to the binding reaction, there was a dose-dependent increase in a second, higher molecular weight complex (←2, Lanes 3-5). These experiments demonstrate that purified Sp1 was capable of forming a stable complex with the half ERE/Sp1 binding site. Additional gel shift assays demonstrated that the more rapidly migrating Sp1-DNA complex had the same mobility as the complex formed with MCF-7 nuclear extracts (Data not shown).

It seemed likely that the formation of the higher order complex in our gel shift experiments represented the simultaneous binding of two Sp1 proteins to the two Sp1 sites and the more rapidly migrating complex represented Sp1 binding to one of the two Sp1 sites. To determine if Sp1 was binding to one or both of the Sp1 sites and whether it displayed any preference in binding to the proximal or the distal Sp1 site, *in vitro* footprinting experiments were carried out. 181 bp DNA fragments, each containing the half ERE/Sp1 binding site, were ^{32}P-labeled on the coding strand and incubated with increasing concentrations of purified Sp1 protein. When 12.5 ng of purified Sp1 was included in the binding reaction, the proximal and distal Sp1 sites were protected (Fig. 7, Lanes 3). Addition of 25 and 37.5 ng of purified Sp1 protein further enhanced protection of the two Sp1 sites (Lanes 4-5). When DNA fragments labeled on the noncoding strand were incubated with increasing amounts of purified Sp1, the proximal Sp1 site was more protected than the distal Sp1 site (Lanes 8-10). This preference for the proximal Sp1 site was also evident in the *in vitro* footprints of the noncoding strand in the presence of MCF-7 nuclear extracts (Fig. 5). Control lanes containing DNA fragments, which had been exposed to DMS (Fig. 7, Lanes 1 and 6) or DNase I (Lanes 2 and 7) in the absence of proteins, were included for reference. These data combined with our gel mobility shift assays supports the idea

that Sp1 binds first to the proximal Sp1 site and then to the distal Sp1 site.

ER enhances Sp1 binding to the half ERE/Sp1binding site. Our *in vitro* binding assays suggested that Sp1 was involved in regulating the PR gene, but left some question about the involvement of ER in this process. From previous studies examining ER-mediated transcription activation, it seemed possible that ER could increase transcription either directly by binding to the ERE half site or indirectly by enhancing Sp1 binding (7-12,41,42). To determine if ER affected protein-DNA complex formation, gel mobility shift assays were carried out. When ^{32}P-labeled oligos, each containing the half ERE/Sp1 binding site, were incubated with 3 ng of purified Sp1 (Fig. 8A, Lane 1), a single gel shifted band was observed. When the amount of purified Sp1 protein was decreased to 0.25 ng, a faint gel shifted band was barely visible (Lane 2). As 5-40 fmol of purified, E_2-occupied ER was added to 0.25 ng purified Sp1 protein, an increase in Sp1 binding was observed (Lanes 3-6). Addition of 40 fmoles of ER increased Sp1 binding 13.1 ($\pm$ 4.2 SE)-fold in three separate experiments. This increased binding was not due to an increase in protein concentration since all reactions contained the same amount of total protein. Interestingly, ER enhanced Sp1 binding, but did not change the mobility of the protein-DNA complex indicating that the ER was not present in the complex. The ability of ER to enhance Sp1 binding without forming a trimeric ER-Sp1-DNA complex in gel mobility shift assays has been noted by others (11,41). Addition of increasing amounts of E_2-occupied ER to the binding reaction also produced a dose-dependent increase in a second, more rapidly migrating complex, which we thought most likely resulted from ER binding to the ERE half site. To confirm which protein-DNA complexes contained ER and Sp1, antibody supershift experiments were carried out. Addition of ER and Sp1 to the binding reaction resulted in the formation of two protein-

DNA complexes (Fig. 8B, Lane 7). The Sp1-specific antibody 1C6 supershifted the more slowly migrating complex, but did not affect the more rapidly migrating complex (Lane 8). The ER-specific antibody H222 decreased the intensity of the more rapidly migrating complex, but did not affect the Sp1-DNA complex (Lane 9). The abilities of these antibodies to interact specifically with the Sp1-DNA and ER-DNA complexes was demonstrated using either purified Sp1 in the absence of ER (Lanes 1-3) or purified ER in the absence of Sp1 (Lanes 4-6). These antibody supershift experiments confirmed that the more slowly migrating complex contained Sp1 and the more rapidly migrating complex contained ER. In contrast to these findings with the E_2-occupied ER, addition of unoccupied ER to levels as high as 100 fmoles, failed to enhance Sp1 binding (Data not shown). Thus, the addition of purified E_2-occupied ER to the binding reaction not only enhanced Sp1 binding, but also resulted in ER binding, presumably, to the ERE half site.

To determine how ER affected Sp1 protection of the half ERE/Sp1 binding site, *in vitro* DNase I footprinting experiments were carried out with purified ER and Sp1 proteins. When 15 ng of purified Sp1 was incubated with the ^{32}P-labeled coding strand, the proximal and distal Sp1 sites were protected (Fig. 9, Lanes 3). Addition of 15 ng Sp1 and 25-100 fmol of purified ER incrementally enhanced the protection of both the proximal and distal Sp1 sites (Lanes 4-6). As suggested from the gel mobility shift assays, the consensus ERE half site was protected in the presence of higher ER concentrations (Lane 6). When DNA fragments labeled with ^{32}P on the noncoding strand were incubated with 15 ng of purified Sp1 and increasing concentrations of purified ER, enhanced protection of both the proximal and distal Sp1 sites and the half ERE was observed (Lanes 9-12). As seen in the *in vitro* footprints with MCF-7 nuclear extracts and with purified Sp1, the proximal Sp1 site on the noncoding strand was more extensively protected than

the distal Sp1 site. The ERE half site was partially protected on the noncoding strand. Control lanes containing DNA fragments, which had been exposed to DMS (Lanes 1 and 7) or DNase I (Lanes 2 and 8) in the absence of proteins, were included for reference.

Purified Sp1 and ER bind differentially to wt and mutant half ERE/Sp1 binding sites. The *in vitro* footprinting experiments reproducibly suggested a preference of Sp1 for the proximal Sp1 site. To determine how each of the Sp1 sites and the ERE half site contributed to protein/DNA complex formation, each of the individual elements was mutated and tested in gel mobility shift assays. Complementary oligos containing the wild type half ERE/Sp1 binding site (wt), or mutations in both Sp1 sites (mP/D), the distal Sp1 site (mD), the proximal Sp1 site (mP), or the ERE half site (mE) were synthesized, annealed, and labeled with ^{32}P. The labeled oligos were combined with purified Sp1 (Fig. 10, Lanes 1-5) or purified Sp1 and ER (Lanes 6-10) and fractionated on nondenaturing gels. Sp1 or Sp1 and ER bound effectively to the wt half ERE/Sp1 site (Lanes 1 and 6). As anticipated, Sp1 did not bind to the oligo containing mutations in both Sp1 sites, in the absence (Lane 2) or in the presence of ER (Lane 7). Sp1 alone or in combination with ER bound to oligos containing a mutation in one of the two Sp1 binding sites, but more protein/DNA complex was formed when the oligo contained an intact proximal Sp1 site (mD; Compare Lanes 3 and 8 with Lanes 4 and 9). These findings corroborate the preferential binding of Sp1 to the proximal Sp1 site observed in the *in vitro* footprinting studies. The ability of ER to bind to oligos containing an intact ERE half site (Lanes 6-9), but not to an oligo containing a mutated ERE half site (Lane 10) further supports the idea that an ER monomer is bound to the ERE half site. When the ERE half site was mutated, increased Sp1/DNA complex formation was

observed (Lanes 5 and 10). The reason for this apparent increase in Sp1 binding is unclear, but it was a reproducible finding.

Discussion

Sequence comparison of the PR gene from different species has been used to identify cis elements that are involved in estrogen-regulated transactivation. The rabbit PR gene contains an imperfect ERE, which overlaps with the translation start site and is capable of conferring estrogen responsiveness to a heterologous promoter in transient transfection assays (43). Although a similar sequence is present in the chicken PR gene (44), no homologous sequence has been identified in the human PR gene (24). A number of studies have suggested that ER and Sp1 may be involved in conferring estrogen responsiveness to the creatine kinase B (7), c-myc (8), retinoic acid receptor α (9), heat shock protein 27 (10,11), cathepsin D (12), and uteroglobin (13) genes. The identification of an ERE half site adjacent to two Sp1 sites in the human PR gene (24) led us to investigate whether this region might be involved in conferring estrogen-responsiveness to the human PR gene. We initiated our studies by examining the endogenous PR gene in MCF-7 cells. Unlike transient transfection assays, which examine the ability of ER to activate transcription of synthetic promoters in supercoiled plasmids, our *in vivo* DNase I footprinting experiments allowed us to examine the endogenous PR gene as it exists in native chromatin and assess whether the half ERE/Sp1 binding site might play a physiological role in gene expression. E_2 treatment of MCF-7 cells did elicit more extensive protection of the half ERE/Sp1 binding site than was observed in the absence of hormone. The enhanced protection of the half ERE/Sp1 binding site seen after 72 hours of hormone treatment, a time when PR mRNA and protein reach maximal levels (16-18,27,39), suggests that sustained protein-DNA interactions are required for maximal production of PR mRNA and protein. Furthermore, the ability of the half ERE/Sp1 binding site

to enhance transcription of a CAT reporter plasmid in the presence of E_2 suggests that this region is involved in estrogen-responsiveness of the PR A promoter.

A role for Sp1 in regulating expression of the PR gene

Sp1 was originally described as a trans acting factor that bound to a GC box (5'GGGCGG3') and activated transcription of the SV40 promoter (45,46). Subsequent comparison of numerous Sp1 binding sites led to the identification of a higher affintiy, consensus Sp1 site, 5'GGGGCGGGGC3' (47) and the discovery that sequences, which varied from this consensus sequence, displayed decreased affinities for Sp1. While both of the Sp1 sites in the human PR half ERE/Sp1 binding site contain the GC box motif, only the proximal Sp1 site contains the 10 bp consensus Sp1 sequence (Fig. 1). The increased affinity of Sp1 for the 10 bp proximal Sp1 site, when compared to the distal Sp1 site, was repeatedly observed in our *in vitro* footprinting assays and was most obvious on the noncoding strand (Figs. 5, 7, and 9). Gel mobility shift assays carried out with oligos containing mutations in the proximal or distal Sp1 site confirmed Sp1's preference for the proximal Sp1 site (Fig. 10). Interestingly, the centers of the two GC boxes present in the half ERE/Sp1 binding site are separated by 10 basepairs or one turn of the DNA helix ($Sp1_D$ +580 to +585, $Sp1_P$ +590 to +595). The periodicity of these elements could either favor interaction between adjacent DNA-bound proteins resulting in cooperative binding or stericly hinder binding of two Sp1 proteins. Our gel mobility shift and *in vitro* DNase I footprinting assays provided evidence for additive, not cooperative, binding of Sp1 to these sites and indicate that Sp1 binds first to the proximal Sp1 site and then to the distal Sp1 site.

A role for ER in regulating expression of the PR gene

The Sp1 sites in the endogenous PR A promoter were more protected after treatment of MCF-7 cells with E_2 in our *in vivo* footprinting experiments and E_2-occupied, but not unoccupied ER, effectively enhanced Sp1 binding to the two Sp1 sites in the PR A promoter in our *in vitro* binding assays. These findings suggest that an E_2-induced change in receptor conformation may be required for ER-enhanced Sp1 binding. Although we and others have been unable to detect ER interaction with the DNA-bound Sp1 in gel mobility shift assays, direct ER-Sp1 interaction has been documented in immunoprecipitation and GST pulldown experiments (11,41). This ability of the E_2-occupied ER to enhance Sp1 binding to DNA provides a mechanism by which estrogen could regulate genes that contain Sp1 sites.

Another way that estrogen might affect PR gene expression is through direct binding of the receptor to the ERE half site. The ERE was protected in our *in vitro* footprinting experiments with ER and Sp1, but not with Sp1 alone demonstrating that the ER did bind to the ERE half site. Likewise, gel mobility shift experiments carried out with purified ER alone or in combination with Sp1 indicated that the ER bound surprisingly well to the ERE half site and formed a stable protein-DNA complex that was capable of withstanding the extensive periods of electorphoresis required for gel mobility shift experiments. Furthermore, the ERE was protected in our *in vivo* footprinting experiments after treatment of MCF-7 cells with E_2 suggesting that the ERE is involved in regulation of the endogenous gene. Although we were unable to detect protection of the ERE half site in our *in vitro* binding assays using MCF-7 nuclear extracts, the level of ER in these extracts (0.42 fmoles/μg protein) was significantly lower than the level present in an intact cell nucleus. Assuming a nuclear radius of 6 μm and 150,000 ER sites per cell (48), the ER

concentration in an MCF-7 nucleus would be 273 nM. These ER concentrations are significantly higher than the 0.25 - 2 nM concentrations used in our *in vitro* binding assays and would most likely favor ER binding to the ERE half site. The 10 bp separating the ERE half site and the distal Sp1 binding site would place the ER on the same side of the DNA helix as the DNA-bound Sp1 proteins and could help to foster protein-protein interactions.

We have considered only ERα in our studies since MCF-7 cells express high levels of ERα, but do not express ERβ (48,49). Although we have not ruled out the possibility that another nuclear protein might bind to the ERE half site, the high levels of nuclear ER, the differential protection of the ERE half site in the presence and absence of E_2, and the demonstrated ability of ER to bind to the ERE half site *in vitro* suggest that it is most likely the ER that interacts with this site *in vivo* and helps to regulate transcription of the PR A promoter.

Regulation of the PR A Promoter in MCF-7 Cells

Our studies support the idea that ER and Sp1 are involved in estrogen-regulated expression of the human PR A promoter. The protection of nucleotides flanking the half ERE/Sp1 binding site in our *in vivo* footprinting experiment suggests that other proteins are associated with the promoter and are involved in transcription activation. Interestingly, the E_2-occupied ER, but not the unoccupied ER, interacts with a number of coactivator proteins, which participate in transcription activation and chromatin remodeling (50-58). The recruitment of these proteins to the DNA-bound, liganded receptor could account for protection of sequences flanking the half ERE/Sp1 binding site and serve as the initiating event in the formation of an active transcription complex.

While models of DNA are typically drawn in a linear array, the packaging of DNA and protein into the nucleus of a cell requires tremendous compaction. This compaction could facilitate interaction between trans acting factors bound to more distant cis elements. Thus, the association of upstream activators such as ER and Sp1 with factors bound to downstream elements could be fostered. In fact, both ER and Sp1 are known to directly associate with TFIID components. ER interacts with TBP, TFIIB, and $TAF_{II}30$ (59-61) and Sp1 interacts with TBP, $TAF_{II}130$, and $TAF_{II}55$ (62-65). The interaction of ER and Sp1 with TBP and its associated proteins could foster formation of a protein-protein network that helps to establish an active transcription complex. Furthermore, the E_2-dependent recruitment of coactivators such as CBP/p300, which can function as a histone acetyltransferase (51), could help remodel chromatin in the PR A promoter and enhance formation of an interconnected protein-protein and protein-DNA network involved in activation of the human PR gene.

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Abbreviations

ERE, estrogen response element; E_2, 17β-estradiol; ER, estrogen receptor; PR, progesterone receptor; CHO, Chinese hamster ovary; LMPCR, ligation mediated polymerase chain reaction; CAT, chloramphenicol acetyl transferase; DMS, dimethylsulfate

Figure Legends

Figure 1. Sequence of the half ERE/Sp1 binding site. The sequence of the half ERE/Sp1 binding site in the PR A promoter originally reported by Kastner et al (24) is shown.

Figure 2. *In vivo* DNase I footprinting of the endogenous PR gene in MCF-7 cells. MCF-7 cells were maintained in serum-free medium for five days, treated with ethanol control (0 h E_2) or 1 nM E_2 for 2 or 72 hours, and then exposed to DNase I. Genomic DNA was isolated and used in *in vivo* LMPCR footprinting. Naked genomic DNA, which had been treated *in vitro* with either DMS (G) or DNase I (V_t), were included as references. The locations of the proximal Sp1 site ($Sp1_P$), distal Sp1 site ($Sp1_D$) and ERE half site are indicated.

Figure 3. Estrogen-enhanced activity of a plasmid containing the half ERE/Sp1 binding site. CHO cells were transfected with TATA CAT or ERE/Sp1-TATA CAT reporter plasmid, hER expression plasmid, β-galactosidase expression plasmid and pTZ nonspecific DNA using the calcium phosphate coprecipitation method as described in Experimental Procedures. Cells were treated with ethanol vehicle or 10 nM E_2. Data represent the average of 9 independent experiments. Values are presented as the mean ± SEM.

Figure 4. Binding of MCF-7 Sp1 protein to the half ERE/Sp1 site. ^{32}P-labeled oligos containing the half ERE/Sp1 binding site were incubated with nuclear extracts from E_2-treated MCF-7 cells. The ER-specific antibody H222 (ER Ab) or the Sp1 specific antibody IC6 (Sp1 Ab) was added to

the binding reaction as indicated. The ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography.

Figure 5. *In vitro* footprinting of the half ERE/Sp1 binding site with MCF-7 nuclear extracts. 181bp DNA fragments containing the half ERE/Sp1 binding site were end-labeled on either the coding and noncoding strand and incubated with increasing concentrations of nuclear extract from E_2-treated MCF-7 cells (Lanes 3-5 and 8-10). The binding reactions were subjected to limited DNase I digestion and the cleaved DNA fragments were fractionated on a denaturing gel. Naked genomic DNA samples, which had been treated *in vitro* with either DMS (Lanes 1 and 6) or DNase I (Lanes 2 and 7), were included as references. The locations of the proximal Sp1 site ($Sp1_P$), distal Sp1 site ($Sp1_D$) and ERE half site are indicated.

Figure 6. Gel mobility shift assay of half ERE/Sp1 binding site-containing oligos and purified Sp1 protein. ^{32}P-labeled oligos containing the half ERE/Sp1 binding site were incubated with increasing concentrations of purified Sp1 protein and fractionated on a nondenaturing gel. The locations of the more rapidly (← 1) and more slowly (← 2) migrating Sp1/DNA complexes are indicated. The complexed and free DNA were visualized by autoradiography.

Figure 7. *In vitro* footprinting of the half ERE/Sp1 binding site with purified Sp1. 181bp DNA fragments containing the half ERE/Sp1 binding site and flanking regions were end-labeled on either the coding and noncoding strands and incubated with increasing concentrations of purified Sp1 protein (Lanes 3-5 and 8-10). The binding reactions were subjected to limited DNase I

digestion and the cleaved DNA fragments were fractionated on a denaturing gel. Naked genomic DNA samples, which had been treated *in vitro* with either DMS (Lanes 1 and 6) or DNase I (Lanes 2 and 7), were included as references. The locations of the proximal Sp1 site ($Sp1_P$), distal Sp1 site ($Sp1_D$) and ERE half site are indicated.

Figure 8. ER-enhanced binding of Sp1 to the half ERE/Sp1 binding site. (Panel A) ^{32}P-labeled oligos containing the half ERE/Sp1 binding site were incubated with 3ng (Lane 1) or 0.25ng of purified Sp1(Lanes 2-6) and 5,10, 20 or 40 fmoles of purified ER (Lane 3-6). ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography. (Panel B) ^{32}P-labeled oligos containing the half ERE/Sp1 binding site were incubated with purified Sp1 (Lanes 1-3), purified ER (Lanes 4-6) or purified Sp1 and ER (Lanes 7-9). The ER-specific antibody H222 (ER Ab) or the Sp1 specific antibody IC6 (Sp1 Ab) were added to the binding reaction as indicated. ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography.

Figure 9. *In vitro* DNase I footprinting of the half ERE/Sp1 binding site with purified Sp1 and ER. DNA fragments containing the half ERE/Sp1 binding site and flanking regions, were end-labeled on either the coding and noncoding strands and incubated with 15ng of purified Sp1 protein (Lanes 3-6 and 9-12) and 25, 50 or 100 fmoles of purified ER (Lanes 4-6 and 10-12). The binding reactions were subjected to limited DNase I digestion and the cleaved DNA fragments were fractionated on a denaturing gel. Naked genomic DNA samples, which had been treated *in vitro* with either DMS (Lanes 1 and 7) or DNase I (Lanes 2 and 8), were included as references.

The locations of the proximal Sp1 site ($Sp1_P$), distal Sp1 site ($Sp1_D$) and ERE half site are indicated.

Figure 10. Interaction of purified Sp1 and ER with wild type and mutant half ERE/Sp1 binding sites. ^{32}P-labeled oligos containing the wild type half ERE/Sp1 binding site (wt), or mutations in both Sp1 binding sites (mP/D), the distal Sp1 binding site (mD), the proximal Sp1 binding site (mP), or the ERE half site (mE) were incubated with 3ng of purified Sp1 (Lanes 1-5) or 3ng of purified Sp1 and 10 fmoles of purified ER (Lane 6-10). The ^{32}P-labeled oligos were fractionated on a nondenaturing gel and visualized by autoradiography.

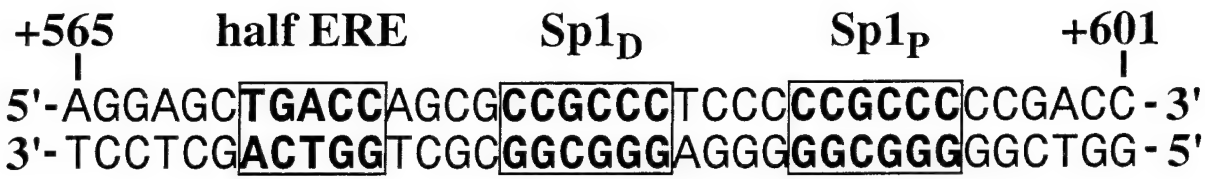

+565
half ERE
Sp1D
Sp1P
+601
5'-AGGAGCTGACCAGCGCCGCCCTCCCCCGCCCCCGACC-3'
3'-TCCTCGACTGGTCGCGGCGGGAGGGGGCGGGGGCTGG-5'

Figure 1

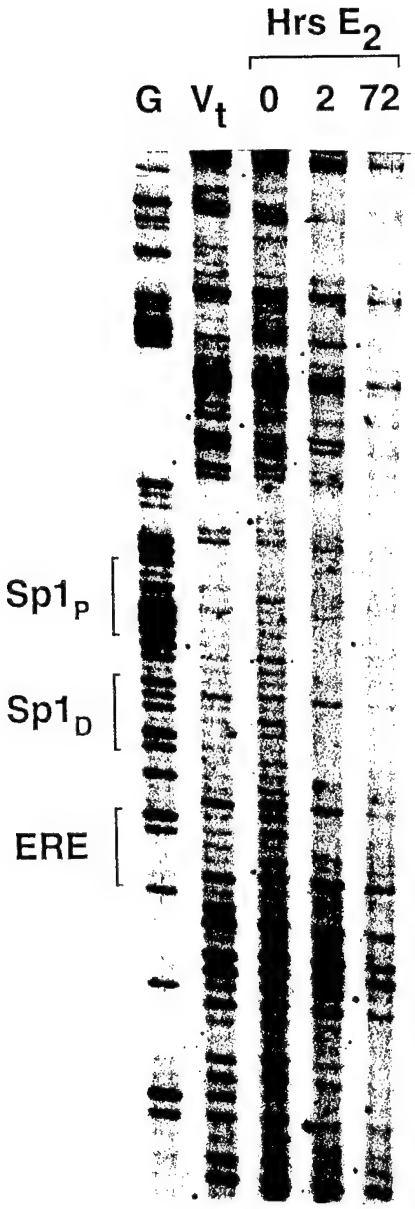


Figure 2

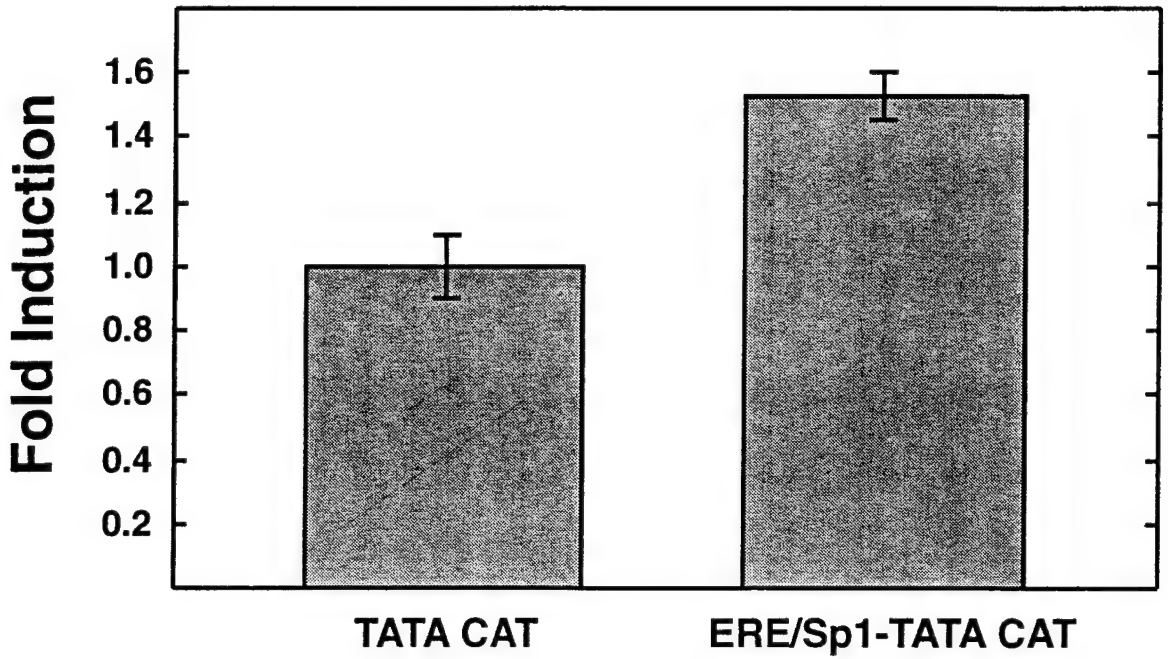


Figure 3

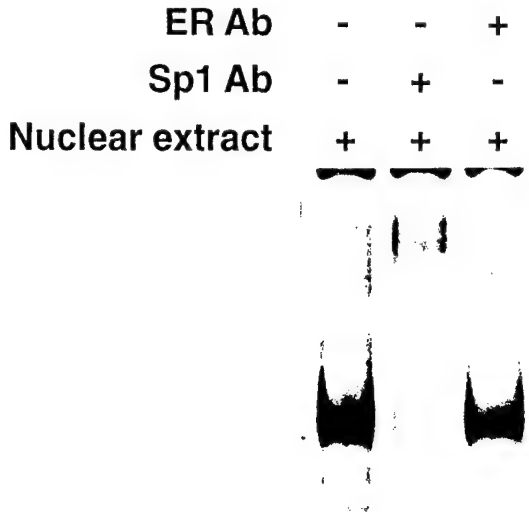


Figure 4

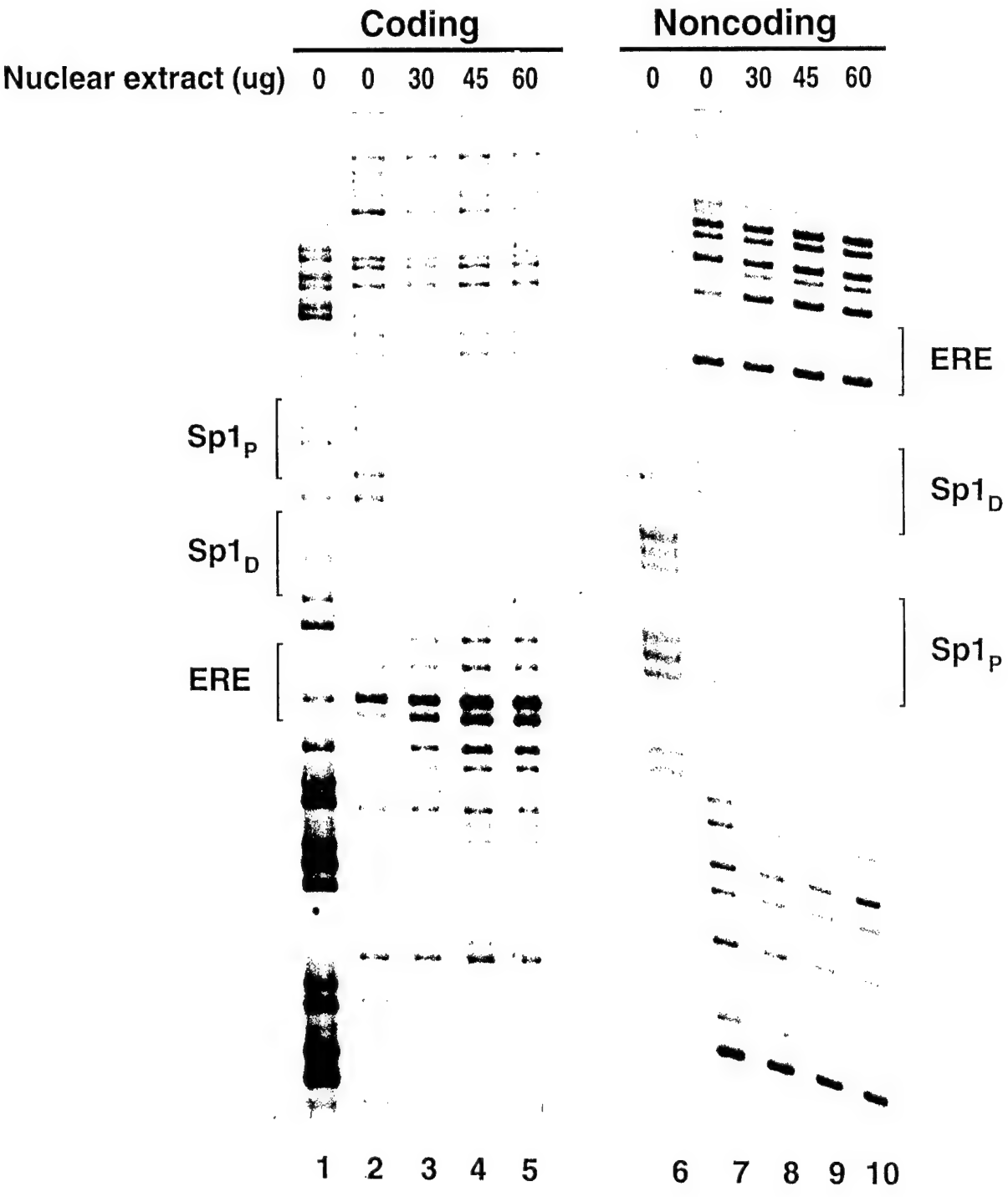
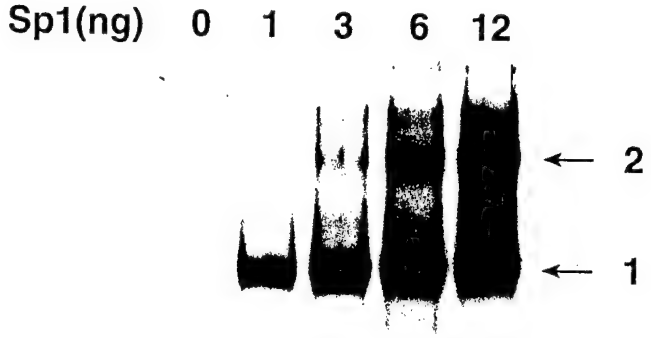

Coding
Noncoding
Nuclear extract (ug)
0 0 30 45 60
0 0 30 45 60
ERE
Sp1P
Sp1D
Sp1D
Sp1P
ERE
1 2 3 4 5
6 7 8 9 10

Figure 5



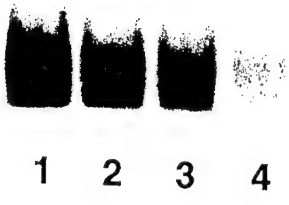


Figure 6

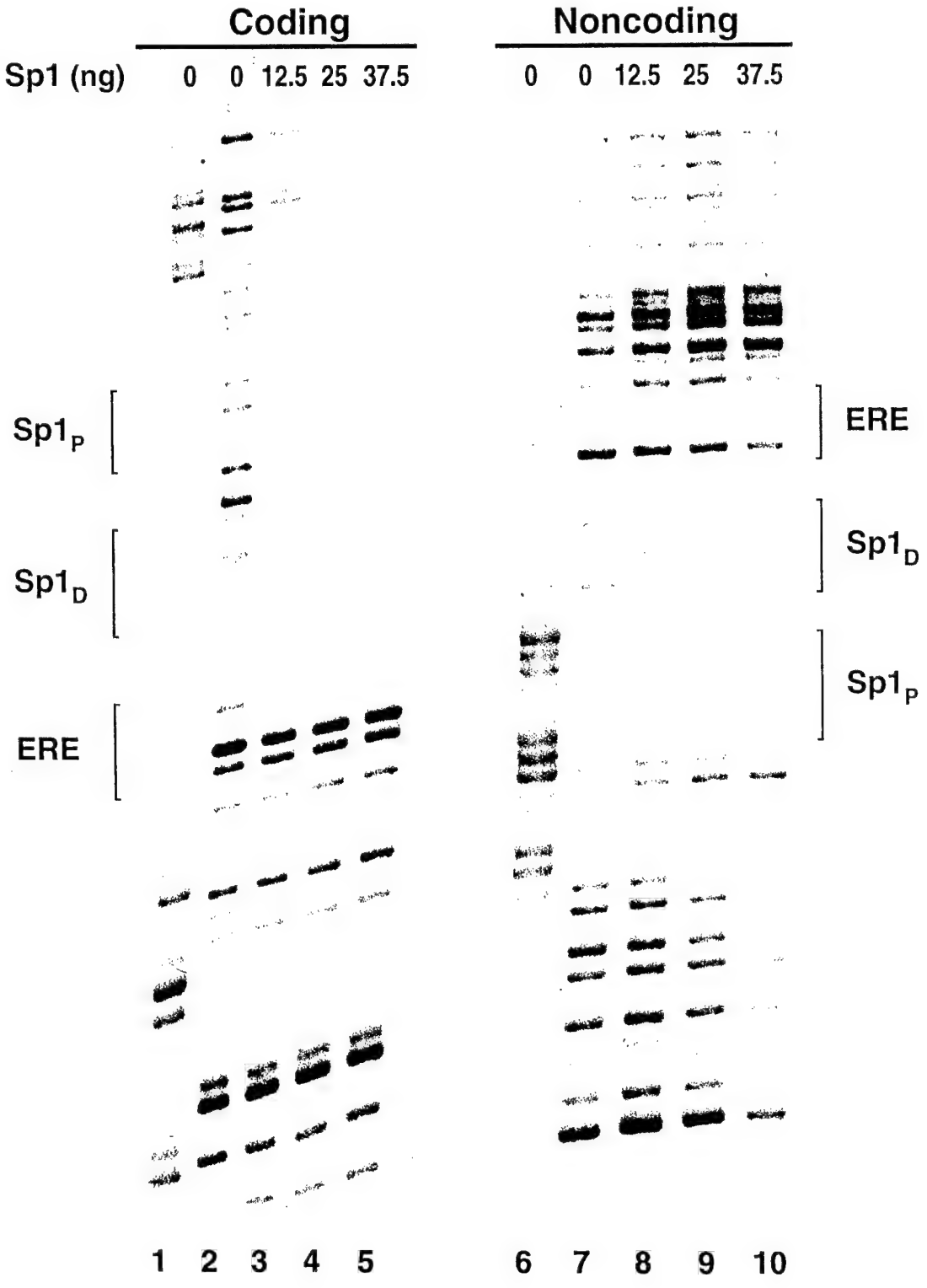

Coding
Noncoding
Sp1 (ng)
0 0 12.5 25 37.5
0 0 12.5 25 37.5
Sp1P
Sp1D
ERE
ERE
Sp1D
Sp1P
1 2 3 4 5
6 7 8 9 10

Figure 7

A

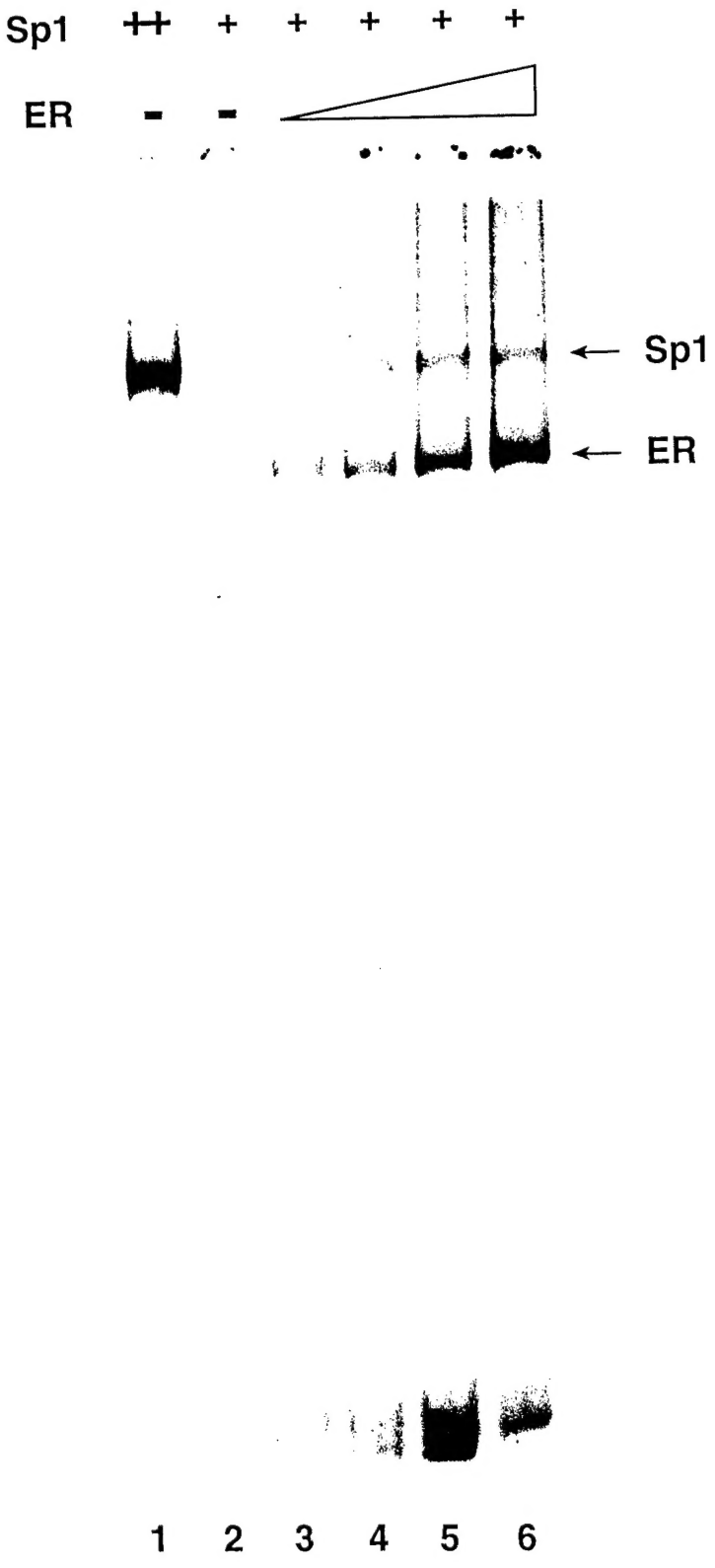


Figure 8A

B

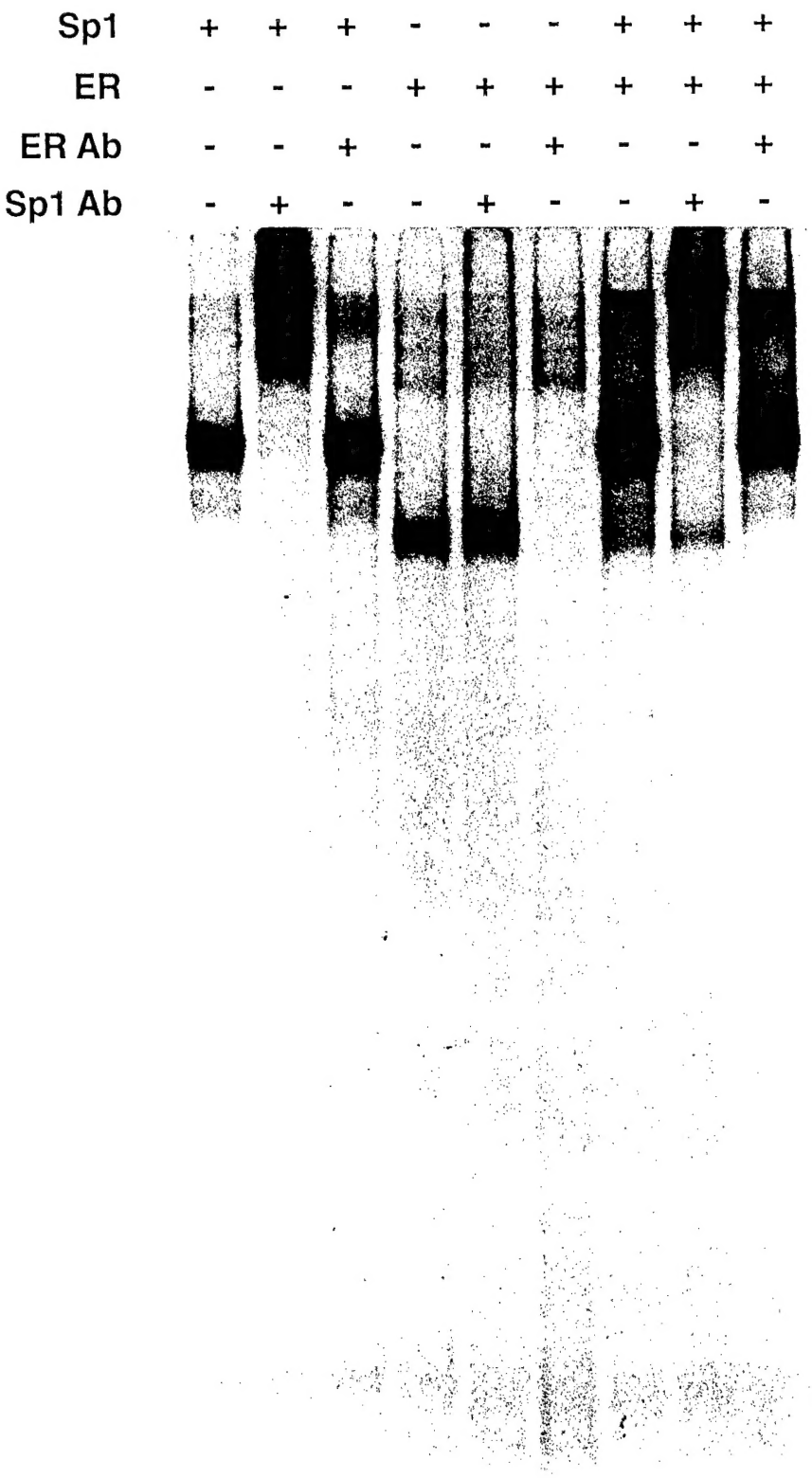


Figure 8B

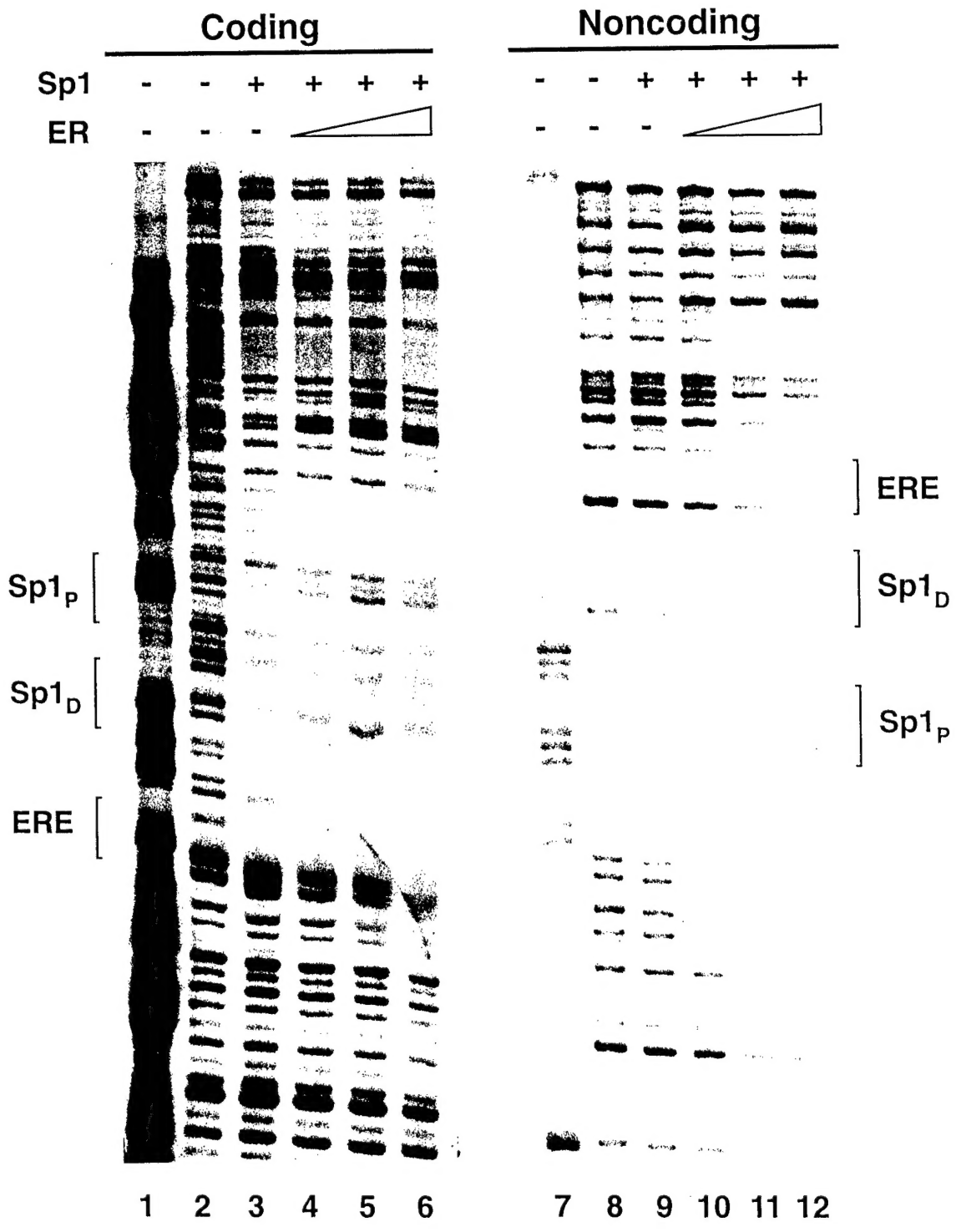

Coding
Noncoding
Sp1
ER
- - + + + +
- - - + + +
Sp1P
Sp1D
ERE
ERE
Sp1D
Sp1P
1 2 3 4 5 6 7 8 9 10 11 12

Figure 9

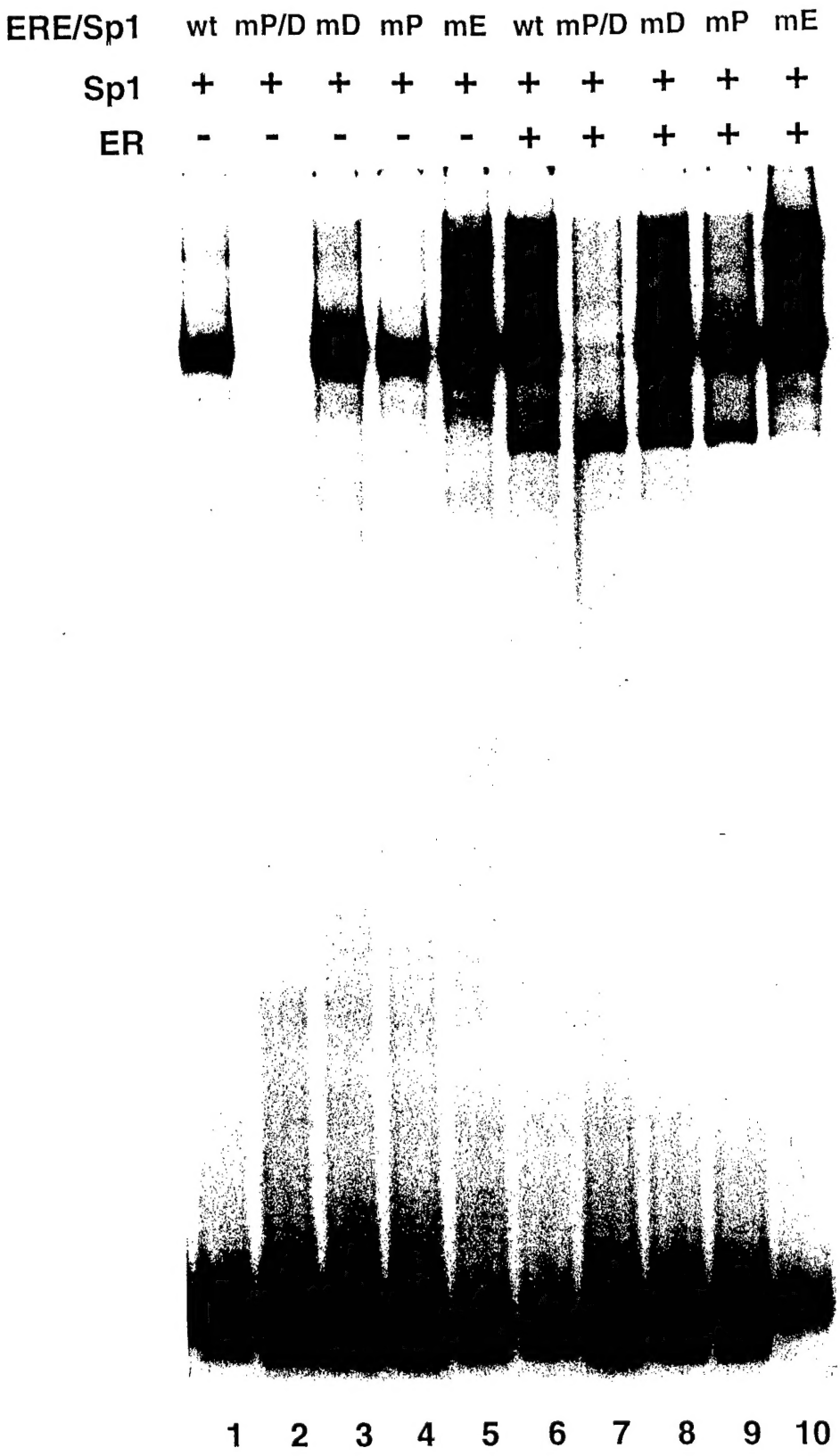


Figure 10